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(57) Abstract

Methods, apparatus and systems for distinguishing various fluorophores based on their fluorescence lifetimes. The techniques of the present invention use modulated radiation to irradiate fluorophores in a detection region. A fluorescence detector outputs a signal proportional to the detected fluorescence emissions, and a processor analyzes the proportional signal to determine fluorescence lifetimes. If the excitation source emits, or is modulated to emit, excitation pulses, the processor can measure the decay time directly; if the excitation source emits, or is modulated to emit, a sinusoidally varying excitation signal, the processor can determine the fluorescence lifetimes by measuring the phase difference or the demodulation relative to an excitation modulation reference signal. A method is provided for identifying components of a mixture by labeling the individual components with fluorescent agents having different fluorescence lifetimes. The components are subsequently separated, fluorescent labels detected and their lifetimes measured. Based on the measured fluorescent lifetimes, the components of mixtures of small organic molecules, polymers, peptides, saccharides and nucleic acids can be identified.

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METHODS AND SYSTEMS FOR SEQUENCING DNA BY DISTINGUISHING THE DECAY TIMES OF FLUORESCENT PROBES

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of USSN 09/132,554, filed August 11, 1998, entitled "METHODS AND SYSTEMS FOR SEQUENCING DNA BY DISTINGUISHING THE DECAY TIMES OF FLUORESCENT PROBES" by Morten J. Jensen and is also a continuation-in-part of 09/213,297, filed December 15, 1998, entitled "DNA SEQUENCING USING MULTIPLE FLUORESCENT LABELS BEING DISTINGUISHABLE BY THEIR DECAY TIMES" by Morten J. Jensen and J. Wallace Parce, which was a non-provisional filing of 60/122,064, filed August 11, 1998. The 60/122,064 application was converted by petition from regular U.S. utility filing USSN 09/132,181 to the provisional 60/122,064 application. The present application claims priority to each of these prior applications, which are also incorporated herein by reference.

FIELD OF THE INVENTION

The present invention provides for the analysis of mixtures of compounds. More particularly, the present invention involves tagging individual compounds with unique fluorescent markers having different fluorescence lifetimes. The analysis of the mixture is then accomplished by distinguishing individual compounds by their unique fluorescence lifetime, using a new apparatus as set forth below.

BACKGROUND OF THE INVENTION

Many fields, including organic chemistry, forensics, medical diagnosis and molecular biology need safe, efficient and cost-effective methods for identifying compounds of interest within a mixture of compounds. Mixtures of compounds frequently arise as the product of an organic synthetic cycle, during the isolation of a product of biological origin and during the chemical or enzymatic sequencing of polymeric compounds such as polypeptides, proteins, polysaccharides and nucleic acids.

For example, accurately determining nucleic acid base sequence is a prerequisite to further understanding the structure and function of the proteins produced by the encoded information. One such method, DNA sequencing, involves determining

the order in which the nucleic acid bases are arranged within a length of DNA. Two DNA sequencing techniques which are widely known, and in current use, are the chemical degradation procedure according to Maxam and Gilbert (*Proc. Natl. Acad. Sci. USA* **74**:560 (1977)) and the enzymatic dideoxy chain termination method of Sanger *et al.* (*Proc. Natl. Acad. Sci. USA* **74**:5463 (1977)). Additionally, *Current Protocols in Molecular Biology*, F.M. Ausubel *et al.*, eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (e.g. Supplement 37, current through 1997) (Ausubel), particularly Chapter 7, provides a description of DNA sequencing in general and discusses various DNA sequencing techniques.

Traditional methods of DNA sequencing utilize a radiolabeled oligonucleotide primer to synthesize a nucleic acid having a sequence complementary to the sequence under analysis. Alternatively, a radiolabeled nucleotide is incorporated directly into the growing nucleic acid strand. Following synthesis, the radioactive nucleic acids are separated by a method such as gel electrophoresis and the positions of the nucleic acids are visualized by autoradiography. Although this technique provides sensitive detection, the use of radioisotopes and autoradiography requires extended exposure times and presents waste disposal problems.

Fluorescent-labeled oligonucleotide primers have been used in place of radiolabeled primers for sensitive detection of DNA fragments (*see, e.g.*, U.S. Pat. No. 4,855,225 to Smith *et al.*). Additionally, DNA sequencing products can be labeled with fluorescent dideoxynucleotides (U.S. Pat. No. 5,047,519 to Prober *et al.*) or by the direct incorporation of a fluorescent labeled deoxynucleotide (Voss *et al. Nucl. Acids Res.* **17**:2517 (1989)). As currently practiced, fluorescent sequencing reactions circumvent many of the problems associated with the use of radionuclides.

In an attempt to increase laboratory throughput and to further decrease exposure of laboratory workers to harmful reagents, various strategies have been developed. For example, robotic introduction of fluids onto microtiter plates is commonly performed to speed mixing of reagents and to enhance experimental throughput. More recently, microscale devices for high throughput mixing and assaying of small fluid volumes have been developed. For example, USSN 08/761,575 "HIGH THROUGHPUT SCREENING ASSAY SYSTEMS IN MICROSCALE FLUIDIC DEVICES" by Parce *et al.* provides pioneering technology related to microscale fluidic devices, especially including electrokinetic devices. The devices are generally suitable

for assays utilizing fluorophores which relate to the interaction of biological and chemical species, including enzymes and substrates, ligands and ligand binders, receptors and ligands, antibodies and antibody ligands, as well as many other assays. Because the devices provide the ability to mix fluidic reagents and assay mixing results in a single continuous process, and because minute amounts of reagents can be assayed, these microscale devices represent a fundamental advance for laboratory science.

The application of fluorogenic and non-fluorogenic assays utilizing fluorescent labels in flowing microfluidic systems are provided, e.g., in Kopf-Sill *et al.*, WO98/56956 "APPARATUS AND METHODS FOR CORRECTING FOR VARIABLE VELOCITY IN MICROFLUIDIC SYSTEMS," filed June 8, 1998. A fluorogenic assay is an assay in which a product of the assay emits a label distinct from those of the reactants of the assay. A non-fluorogenic assay is an assay in which the mobility of a product differs from those of labeled reactants (*e.g.*, in a flowing electrokinetic system), but the emitted label is still essentially the same as the label found on a reactant. Detection of non-fluorogenic assay products is possible e.g., in an electroosmotically driven microfluidic device using periodic injections of reaction mixture into a separation channel, in which reactants and products are separated by electrophoresis due to changes in the electrophoretic mobility resulting from the reaction (*see* also, A. R. Kopf-Sill, T. Nikiforov, L. Bousse, R. Nagel, & J. W. Parce, "Complexity and performance of on-chip biochemical assays," in Proceedings of Micro- and Nanofabricated Electro-Optical Mechanical Systems for Biomedical and Environmental Applications, SPIE, Vol. 2978, San Jose, California, February 1997 p. 172-179).

Closed-loop biochemical microfluidic devices especially adapted to sequencing nucleic acids, as well as for high-throughput screening are described in WO98/45481, entitled "CLOSED-LOOP BIOCHEMICAL ANALYZERS" by Knapp *et al.* In brief, in the integrated microfluidic systems described, it is possible to use the results of a first sequencing reaction, or set of sequencing reactions, to select appropriate reagents, reactants, products, or the like, for additional analysis. For example, the results of a first sequencing reaction can be used to select primers, templates, or the like, for additional sequencing, or to select related families of compounds for screening in high-throughput assay methods. These primers or templates are then accessed by the system and the process continues.

In the above applications, fluorescent dyes are commonly detected/distinguished by their emission spectra. In particular, because different fluorescent dyes are typically excited at different wavelengths (or frequencies) and/or emit at different wavelengths (or frequencies), appropriately selected fluorescent dyes can be distinguished by measuring the corresponding wavelengths (or frequencies). This distinguishing characteristic has led to methodologies and techniques for sequencing nucleic acids by detecting the emission spectra of labeled nucleic acid samples as noted above. For example, according to one method, a fluorescent labeled oligonucleotide is used to synthesize nucleic acid samples having a sequence complementary to the sequence under analysis. Following synthesis, the fluorescent labeled nucleic acid samples are separated by a method such as gel electrophoresis and the positions of the nucleic acids are determined by spectrally identifying the order of the fluorescent labels. See, e.g., U.S. Patent No. 5,171,534, the disclosure of which is hereby incorporated by reference in its entirety.

Such multicolor spectral detection techniques and devices, however, tend to be quite costly and cumbersome, as they typically require a separate filter and detector for each color to be detected. In the case of DNA sequencing techniques using a different fluorescent label or probe for each of the four nucleotides, four filters and four detectors are required. Also, if different labeling techniques are used with one device, many different labels may need to be used for each technique. Accordingly, an appropriate number of additional filters would be required for detecting the various labels. Additionally, depending on the spectral properties of the various fluorescent labels used, background light may interfere with the detection of the fluorescent emission spectra. Unfortunately, additional filters and/or optics are typically required to prevent background light close to the fluorescent emission wavelengths from interfering with the measurements, thereby adding to the cost, size and complexity of the detection system.

Although, as noted, sequencing and other assay methods that utilize fluorescent markers often represent, in many ways, an improvement over methods that utilize radioactive isotopes, current fluorescent methodologies are hampered by certain deficiencies. For example, in order to identify individual nucleotides, each nucleotide must bear a fluorescent marker that has by a unique absorbance and/or emission spectrum with a different absorbance or emission maximum. Thus, to clearly distinguish the individual nucleotides based upon the fluorescence spectrum of their tags, the absorbance

or emission maxima of each tag must be clearly resolved from those of every other tag. Further, fluorescence must be monitored at a number of different wavelengths in order to detect each of the maxima and a filtering system must be employed. This is cumbersome and increases the expense of the instrumentation. This situation is additionally complicated by the dependence of the absorption or emission maxima for a compound upon the environment surrounding that compound.

Thus, a method of detecting individual fluorescently labeled compounds within a mixture of compounds which relied on a characteristic of the fluorescent moiety other than its absorption and/or emission spectrum (*e.g.*, maxima) would represent a significant advance in the art. The present invention provides such a method and apparatus for practicing the methods.

SUMMARY OF THE INVENTION

It has now been discovered that individual members of a mixture can be distinguished and identified through the selective use of a set of fluorescent labels displaying a range of unique fluorescence lifetimes. This method is versatile and it can be practiced with a wide range of separation modalities, fluorescent markers and labeling chemistries. Further, because it detects fluorescence lifetimes, rather than fluorescence emission or excitation maxima, this method is able to resolve a mixture containing several fluorescent species with overlapping fluorescent excitation and/or emission maxima. The present invention provides novel methods, apparatus and systems for distinguishing various fluorescent dyes based on their fluorescence lifetimes or decay times. The present invention further provides novel methods, apparatus and systems for determining the sequence of nucleotides in a nucleic acid sample by measuring and distinguishing the fluorescence lifetimes of fluorescent labels bound to the nucleotides in the nucleic acid sample.

Thus, in a one aspect, the present invention provides a method of distinguishing between a plurality of fluorescent species. The fluorescent species are first electrokinetically transported through a microfluidic channel. The fluorescent species are then excited by irradiating them with electomagnetic energy. The excitation can occur either during the transporting or at the completion of the transporting. Following this excitation, the fluorescent molecules are allowed to return to their ground state. This process is accompanied by a fluorescence emission which is characteristic for each

fluorescent species and which is characterized by a temporal duration referred to as the fluorescence lifetime.

The lifetimes for each of the fluorescent labels is detected at a detecting station and the labeled species are identified by measuring the characteristic fluorescence lifetime of the label to which they are conjugated. It will be apparent to one of skill in the art that the present method can be practiced with any of an array of detecting station configurations. The detection station can include, for example, a laser or pulse lamp to excite the fluorescent species. Additionally, any useful configuration of lenses, prisms, mirrors, diffraction gratings, monochromators and the like can be used to practice the present invention. Useful detectors include fast, high sensitivity optical detectors like PMT, Avalanche Photo Diodes and Photo Diodes. The detector can be coupled to a digital computer that receives incoming data from the detector and processes it into a form useful for distinguishing between the lifetimes of the labels.

By detecting the fluorescence emission and measuring its lifetime for each of the fluorescent species in a mixture, the different fluorescent species present in the mixture can be detected and identified. Single or overlapping emissions that are composed of species with different lifetimes can be mathematically resolved into individual lifetimes, allowing the identification of the individual fluorescent constituents contributing to the emission.

The method is generally useful for the detection and identification of a broad range of compounds. It can be used to identify individual molecules which range in size and functionality from small organic, inorganic or organometallic molecules to proteins, including enzymes, antibodies and the like. The method of the invention can also be used to characterize and identify synthetic polymers and oligomers. These polymers and oligomers find utility in diverse fields of endeavor, including industrial applications, mechanical applications, drugs, foodstuffs and textiles. Synthetic, natural and modified polymers and oligomers of biomolecules such as amino acids, nucleic acids and saccharides can also be identified using the method of the invention.

Thus, in an additional aspect, the present invention provides a method of sequencing a nucleic acid polymer of interest. In this aspect of the invention, the method comprises performing a sequencing reaction on the nucleic acid polymer. Any of the sequencing reactions known in the art is appropriate for use in this aspect. Thus, methods

which chemically or enzymatically degrade or synthesize nucleic acids are of use in practicing the present invention.

During the course of the sequencing reaction, one or more fluorescent labels is incorporated into either the nucleic acid being sequenced or a sequence complementary to the nucleic acid being sequenced. Several methods for performing this incorporating are known in the art. A non-limiting list includes the Sanger, Sanger dideoxy and Maxam-Gilbert sequencing methodologies.

Sequencing reaction mixtures that are useful in practicing the present invention include those that contain the nucleic acid to be sequenced and a fluorescent label. The fluorescent label is attached to a first labeled nucleic acid selected from the group consisting of labeled nucleic acids, labeled nucleic acid polymers and combinations thereof. The fluorescent species are electrokinetically transported through a microfluidic channel to resolve or partially resolve the mixture into separate components.

As discussed above, the fluorescent label will, following excitation, emit electromagnetic energy that is characterized by a distinct and detectable lifetime. When more than one fluorescent label is utilized in the sequencing reaction mixture, each of the labels will have a fluorescent lifetime that is distinct from other labels and thereby detectable. The fluorescence emission is detected at a detecting station.

In addition to the above-described methods, the present invention also provides apparatus that are useful, e.g., in practicing the methods of this invention. The apparatus is capable of distinguishing between a plurality of fluorescent species, wherein each of the fluorescent species has a fluorescence emission, the emission having a characteristic fluorescence lifetime.

The apparatus of the invention comprises a microfluidic device that includes at least one microchannel fabricated within the body structure of the device. The fluorescent species flows through the microchannel by means of, for example, pressure, electroosmosis, electrokinesis, capillarity and the like. The microchannel is linked to a detecting station that is capable of detecting the fluorescent species in the microchannel. The signal from the detector is sent to a digital computer that is operably linked to the detector. The digital computer is appropriately configured or programmed to determine the fluorescence lifetimes of the fluorescent species.

Using techniques and apparatus of the present invention, many different fluorescent dyes can be distinguished without the need for a different optical filter for

each dye and without having to change optical filters when dyes are changed, as long as all dyes are excited by a common wavelength. Additionally, the techniques of the present invention avoid many problems with interference from background light and background fluorescence.

In one aspect, the techniques of the present invention use modulated radiation to irradiate fluorophores in a detection region. A beamsplitter element is optionally provided to selectively pass excitation signals to the detection region and to redirect fluorescence emissions toward a fluorescence detector. The fluorescence detector outputs a signal proportional to the fluorescence emissions detected, and a processor analyzes the proportional signal to determine fluorescence lifetimes. If the excitation source emits, or is modulated to emit, excitation pulses, the processor measures the decay time directly; if the excitation source emits, or is modulated to emit, an oscillating excitation signal, the processor determines the fluorescence lifetimes by measuring the phase difference relative to an excitation modulation reference signal. A phase-locked loop (PLL) is preferably used to determine phase differences.

According to an aspect of the invention, an apparatus is provided for use in measuring the fluorescent lifetimes of a plurality of fluorescent labels, where the labels are provided to a detection region of the apparatus. The apparatus typically includes a radiation source. The radiation source emits, or is modulated to emit, radiation for irradiating the detection region, where the radiation excites the fluorescent labels and causes the labels to fluoresce. The apparatus also typically includes a fluorescence detector for detecting the fluorescent emissions from the fluorescent labels in the detection region, where each one of the plurality of fluorescent labels has a different fluorescent lifetime. The apparatus normally has a processor, coupled to the fluorescence detector, for analyzing the fluorescent emissions to determine the fluorescent lifetimes of the labels.

According to another aspect of the invention, an apparatus is provided for use in determining the sequence of nucleotides in a nucleic acid sample having a plurality of overlapping nucleic acid fragments, where each fragment includes one of four different fluorescent labels. Each label has a different fluorescent lifetime, with each of the labels binding to a specific nucleotide. The nucleic acid fragments of the nucleic acid sample are provided to a detection region. The apparatus typically includes a radiation source. The radiation source emits, or is modulated to emit, radiation for irradiating the detection

region, where the radiation excites the fluorescent labels and causes the labels to fluoresce. The apparatus also typically includes a fluorescence detector for detecting the fluorescent emissions from the fluorescent labels in the detection region. The apparatus ordinarily has a processor, coupled to the fluorescence detector, for analyzing the fluorescent emission to determine the fluorescent lifetimes of the labels, thereby determining the sequence of nucleotides in the nucleic acid.

According to yet another aspect of the invention, a system is provided for measuring the fluorescent lifetimes of a plurality of fluorescent labels. The system typically includes a detection region; where the labels are provided to the detection region. The system also typically includes a radiation source. The radiation source emits, or is modulated to emit, radiation for irradiating the detection region, where the radiation excites the fluorescent labels and causes the labels to fluoresce. The system also typically includes detection means for detecting the fluorescent emissions from the fluorescent labels in the detection region; and means, coupled to the fluorescence detector, for analyzing the fluorescent emissions to determine the fluorescent lifetimes of the labels.

According to a further aspect of the invention, a method of measuring the fluorescent lifetimes of a plurality of fluorescent labels is provided. The method typically includes the steps of providing the labels to a detection region and of irradiating the detection region with radiation emitted from a radiation source, where the radiation excites the fluorescent labels and causes the labels to fluoresce. The method also typically includes the steps of detecting the fluorescent emissions of the fluorescent labels using a fluorescence detector, where each one of the plurality of fluorescent labels has a different fluorescent lifetime, and of analyzing the fluorescent emissions using a processor coupled to the fluorescence detector to determine the fluorescent lifetimes of the labels.

Reference to the remaining portions of the specification, including the drawings and claims, will realize other features and advantages of the present invention. Further features and advantages of the present invention, as well as the structure and operation of various embodiments of the present invention, are described in detail below and with respect to the accompanying drawings. In the drawings, like reference numbers indicate identical or functionally similar elements.

Other objects and advantages of the present invention will be apparent from the detailed description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts an example of a microfluidic device for use with certain aspects of the present invention;

Figure 2 illustrates examples of fluorescence emission signals for long and short decay time fluorophores that have been excited by a pulsed excitation signal;

Figure 3 illustrates examples of fluorescence emission signals for long and short decay time fluorophores that have been excited by a sinusoidally modulated excitation signal;

Figure 4 is a block diagram of a fluorescence detection system according to one embodiment of the present invention;

Figure 5 is a block diagram of a fluorescence detection system according to an alternate embodiment of the present invention; and,

Figure 6 is a block diagram of a fluorescence detection system according to another alternate embodiment of the present invention.

DETALED DESCRIPTION

In a first aspect, the present invention provides a method of distinguishing between a plurality of fluorescent species. The fluorescent species are first electrokinetically transported through a microfluidic channel. The fluorescent molecules are then excited by irradiating them with electomagnetic energy. The excitation can occur either during the transporting or at the completion of the transporting. Following this excitation, the fluorescent molecules are allowed to return to their ground state. This process is accompanied by a fluorescence emission which is characteristic for each fluorescent species and which is characterized by a temporal duration referred to as the fluorescence lifetime of that species.

The lifetimes for each of the fluorescent labels is detected at a detecting station and the labeled species are identified by measuring the characteristic fluorescence lifetime of the label to which they are conjugated. It will be apparent to one of skill in the art that the present method can be practiced with any of an array of detecting station configurations. The detection station can include, for example, a laser or pulse lamp to excite the fluorescent species. Additionally, any useful configuration of lenses, prisms, mirrors, diffraction gratings, monochromators and the like can be used to practice the present invention. Useful detectors include fast, high sensitivity optical detectors like

PMT, Avalanche Photo Diodes and Photo Diodes. The detector can be coupled to a digital computer that receives incoming data from the detector and processes it into a form useful for distinguishing between the lifetimes of the fluorescent labels.

By detecting the fluorescence emission and measuring its lifetime for each of the fluorescent species in a mixture, the different fluorescent species present in the mixture can be detected and identified. Single or overlapping emissions that are composed of species with different lifetimes can be mathematically resolved into individual lifetimes, allowing the identification of the individual fluorescent constituents contributing to the emission.

In preferred aspects, the methods and apparatus of the instant invention are used in the detection of fluorescence emission signals from analytical systems employing fluorescence detection in microscale fluidic channels. Examples include, e.g., fused silica capillary systems, i.e., CE, as well as microfluidic devices and systems that incorporate microscale elements such as microfluidic channels. Such systems are generally described in U.S. Patent Application Nos. 08/845,754, filed April 25, 1997, PCT Application Publication No. 98/00231, filed June 24, 1997 and WO98/56956.

As described above, the methods of the present invention are generally carried out in "microfluidic devices" or "microlaboratory systems," which allow for integration of the elements required for performing the assay, automation, and minimal environmental effects on the assay system, e.g., evaporation, contamination, human error, and the like. A number of devices for carrying out the assay methods of the invention are described in substantial detail herein. However, it will be recognized that the specific configuration of these devices will generally vary depending upon the type of assay and/or assay orientation desired. For example, in some embodiments, the screening methods of the invention can be carried out using a microfluidic device having two intersecting channels. For more complex assays or assay orientations, multichannel/intersection devices are optionally employed. The small scale, integratability and self-contained nature of these devices allows for virtually any assay orientation to be realized within the context of the microlaboratory system.

As used herein, the term "microfluidic" generally refers to one or more fluid passages, channels, chambers or conduits which have at least one internal cross-

sectional dimension, e.g., depth, width, length, diameter, etc., that is less than 500μm, and typically between about 0.1 μm and about 500 μm.

In the devices of the present invention, the microscale channels or chambers preferably have at least one cross-sectional dimension between about 0.1 μm and 200 μm, more preferably between about 0.1 μm and 100 μm, and often between about 0.1 μm and 50 μm. Accordingly, the microfluidic devices or systems prepared in accordance with the present invention typically include at least one microscale channel, usually at least two intersecting microscale channels, and often, three or more intersecting channels disposed within a single body structure. Channel intersections may exist in a number of formats, including cross intersections, "T" intersections, or any number of other structures whereby two channels are in fluid communication.

A "microfluidic" channel is a channel (enclosed groove, depression, tube, capillary, etc.) which is adapted to handle small volumes of fluid. In a typical embodiment, the channel is a tube, channel or conduit having at least one subsection with at least one cross-sectional dimension of between about 0.1μm and 500μm, and typically less than 100μm; ordinarily, the channel is closed over a significant portion of its length, having top, bottom and side surfaces. In operation, materials that are being analyzed, e.g., subjected to optical analysis for fluorescence emission signals, in these microscale fluidic systems, are transported along the microscale fluid channels, past a detection point, where a detectable fluorescence emission signal is measured. The signals within these channels typically result from the presence of fluorescent substances therein, e.g., fluorophores that inherently fluoresce, or are made to fluoresce, that are used as indicators of the presence or absence of some material or condition.

The body structure of the microfluidic devices described herein typically comprises an aggregation of two or more layers (which can be fused or bonded) which, when appropriately mated or joined together, form the microfluidic device of the invention, e.g., containing the channels and/or chambers described herein. Typically, the microfluidic devices described herein will comprise a body structure having a top portion, a bottom portion, and an interior portion, wherein the interior portion, or microscale cavity, substantially defines the channels and chambers of the device.

Suitable substrate materials for the body structure are generally selected based upon their compatibility with the conditions present in the particular operation to be

performed by the device. Such conditions can include extremes of pH, temperature, salt concentration, and application of electrical fields. Additionally, substrate materials are also selected for their inertness to critical components of an analysis or synthesis to be carried out by the device. Examples of useful substrate materials include, *e.g.*, glass, quartz and silicon as well as polymeric substrates, *e.g.* plastics, particularly polyacrylates. In the case of conductive or semi-conductive substrates, it is occasionally desirable to include an insulating layer on the substrate. This is particularly important where the device incorporates electrical elements, *e.g.*, electrical fluid direction systems, sensors and the like. In the case of polymeric substrates, the substrate materials may be rigid, semi-rigid, or non-rigid, opaque, semi-opaque or transparent, depending upon the use for which they are intended. For example, devices which include an optical, spectrographic, photographic or visual detection element, will generally be fabricated, at least in part, from transparent materials to allow, or at least, facilitate that detection. Alternatively, transparent windows of, *e.g.*, glass or quartz, are optionally incorporated into the device for these types of detection elements. Additionally, the polymeric materials optionally have linear or branched backbones, and may be crosslinked or non-crosslinked. Examples of polymeric materials include, *e.g.*, polydimethylsiloxanes (PDMS), polyurethane, polyvinylchloride (PVC) polystyrene, polysulfone, polycarbonate and the like.

In one set of preferred aspects, the methods and apparatus of the present invention are used for determining the sequence of nucleotides in a nucleic acid sample wherein the nucleotides are tagged or labeled with fluorescent labels as is well known in the art. For example, according to one common method as discussed above, a fluorescent-labeled oligonucleotide is used to synthesize a nucleic acid having a sequence complementary to the sequence under analysis. Additionally, nucleic acids can be labeled with fluorescent dideoxynucleotides or by the direct incorporation of a fluorescent labeled dideoxynucleotide. Following synthesis, the fluorescent-labeled nucleic acids are separated by a method such as gel electrophoresis and the positions of the nucleic acids are determined by identifying the order of the fluorescent markers or labels using the techniques of the present invention. According to alternate aspects, the present invention is useful for detecting and distinguishing any fluorophore labeled substances (for example, any fluorophore labeled substance in an electrophoretic separation medium or the like) and any fluorescing substances.

As noted above, in microscale analytical systems, a fluorescent material or a fluorescent-labeled material is transported along the microscale channel and past a detection point. Typically, transporting materials within these systems may be carried out by any of a variety of methods. For example, such material transport is optionally carried out through the application of pressures to the materials within the channels, through the incorporation of microscale mechanical pumps, or through the application of electric fields, to move materials through the channels.

In preferred aspects, the above microfluidic systems use electrokinetic transport systems for moving material within the microfluidic channels. As used herein, "electrokinetic material transport systems" include systems which transport and direct materials within an interconnected channel and/or chamber containing structure, through the application of electrical fields to the materials, thereby causing material movement through and among the channel and/or chambers (i.e., cations will move toward the negative electrode, while anions will move toward the positive electrode). Such electrokinetic material transport and direction systems include those systems that rely upon the electrophoretic mobility of charged species within the electric field applied to the structure. Such systems are more particularly referred to as electrophoretic material transport systems. Other electrokinetic material direction and transport systems rely upon the electroosmotic flow of fluid and material within a channel or chamber structure which results from the application of an electric field across such structures. In brief, when a fluid is placed into a channel which has a surface bearing charged functional groups, e.g., hydroxyl groups in etched glass channels or glass microcapillaries, those groups can ionize. In the case of hydroxyl functional groups, this ionization (e.g., at neutral pH), results in the release of protons from the surface and into the fluid, creating a concentration of protons at near the fluid/surface interface, or a positively charged sheath surrounding the bulk fluid in the channel. Application of a voltage gradient across the length of the channel will cause the proton sheath to move in the direction of the voltage drop (i.e., toward the negative electrode). In additional preferred aspects, pressure-driven flow is used to move components in microfluidic channels or channel regions. Additional details on the movement of materials in microfluidic systems are found below.

Figure 1 depicts an example of a microfluidic device for use with certain aspects of the present invention. As shown, the device 100 includes a body structure 102 which has an integrated channel network 104 disposed therein. The body structure 102

includes a plurality of reservoirs 106-128, disposed therein, for holding reagents, sample materials, and the like. Also included is buffer reservoir 130, as well as waste reservoirs 132, 134 and 136. The reagents, samples, etc. are transported from their respective reservoirs, either separately or together with other reagents from other reservoirs into a main channel 138, and along main channel 138 toward waste reservoir 136, past detection zone or window 140. Detection window 140 is typically transparent, and may be comprised of a transparent region of the body structure, or a separate transparent window fabricated into the body structure. Typically, the body structure is itself fabricated from a transparent material, e.g., glass or transparent polymers, thereby obviating the need for a separate transparent region to define the detection window. Microfluidic devices of the sort described above are useful in performing a variety of analyses, such as electrophoretic separation of macromolecules, e.g., nucleic acids, proteins, etc. (see U.S. Application No. 08/845,754, filed April 25, 1997), high throughput screening assays, e.g., in pharmaceutical discovery, and diagnostics, e.g., immunoassays (see, e.g., Published PCT Application WO 98/00231). In general, the above device structures and channel geometries, and an infinite number of other similar device structures and channel geometries can be implemented with various aspects of the present invention.

The individual members of virtually any complex mixture can be distinguished and identified using the method of the invention. Exemplary species include, for example, individual members of compound libraries (*e.g.*, small organic molecules, peptides, nucleic acids) and the products of sequencing reactions. In a presently preferred embodiment, the method is used to distinguish between a plurality of fluorescent products derived from a sequencing reaction performed on a nucleic acid, a peptide or an oligosaccharide. In a further preferred embodiment, the fluorescent products are derived from a dideoxy nucleotide chain termination method sequencing reaction mixture derived from one or more nucleic acids. See, for example, Sanger *et al.*, *Proc. Natl. Acad. Sci. USA* 74: 5463-5467 (1977); U.S. Pat. No. 5,171,534, to Smith *et al.* The means of practicing various embodiments of the present invention will be apparent from the theoretical and practical discussion that follows.

Fluorescence Lifetime

The fluorescence lifetime of a fluorophore in an excited state is defined as the average time the molecule spends in the excited state prior to return to the ground state. Generally, fluorescence lifetime is described by:

$$\tau = 1 / (\Gamma + k) \qquad (1)$$

where τ is the fluorescence lifetime, Γ is the emissive rate constant of the fluorophore and k is the rate constant of radiationless decay.

Because fluorescence emission is a random process, few molecules will emit photons at $t = \tau$. The lifetime τ is merely an average value of the time spent in the excited state. For a single exponential decay, 63% of the molecules will have decayed prior to $t = \tau$ and 37% decay at $t > \tau$.

The lifetime of a particular fluorophore, in the absence of nonradiative processes, is called the intrinsic lifetime:

$$\tau_0 = 1 / \Gamma. \qquad (2)$$

Thus, the relationship between the quantum yield Q and the fluorescence lifetime τ for a particular molecule is:

$$Q = \tau / \tau_0 \qquad (3)$$

Both the quantum yield Q and the fluorescence lifetime τ can be modified by factors which affect either of the rate constants Γ and k. For example, some molecules can be substantially non-fluorescent with a large rate of internal, radiationless conversion. Such molecules typically have low quantum yields and short fluorescence lifetimes. Additionally, scintillation agents, which have high quantum yields as a result of high Γ values, typically have very short fluorescence lifetimes.

Fluorophores

In the present invention, a fluorophore is a substance which itself fluoresces, or is made to fluoresce, or is a fluorescent analogue of an analyte. In principal, any fluorophore now known, or later discovered, can be used with the present invention.

Fluorescent species having lifetimes that fall within a broad range of measurable lifetimes are useful in the present invention. For example, according to aspects of the present invention, the fluorescence lifetimes of fluorophores preferably range from about 0.1 nanoseconds to about 4000 nanoseconds, more preferably from

about 0.1 nanoseconds to about 1000 nanoseconds, and even more preferably from about 0.1 nanoseconds to about 100 nanoseconds. Furthermore, particularly preferred fluorophores have the following characteristics:

a. A fluorescence lifetime of greater than about 15 nanoseconds;

b. An excitation wavelength of greater than about 350 nanometers;

c. A Stoke's shift (a shift to a higher wavelength, or a lower frequency, of the emission relative to the absorption) of greater than about 20 nanometers; and

d. A high absorptivity and quantum yield.

A longer lifetime is advantageous because it is easier to measure and more easily distinguishable from Raleigh scattering (background) effects. Excitation wavelengths greater than 350 nanometers reduce the background interference because most fluorescent substances responsible for background fluorescence in biological samples are excited below 350 nanometers. A greater Stoke's shift also allows for less background interference.

Specific fluorescent compounds which are useful in practicing the present invention include, but are not limited to, dansyl, fluorescein, 8-anilino-1-napthalene sulfonate, pyrene, ethenoadenosine, ethidium bromide prollavine monosemicarbazide, p-terphenyl, 2,5-diphenyl-1,3,4-oxadiazole, 2,5-diphenyloxazole, p-bis[2-(5-phenyloxazolyl)]benzene, 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benxene, lanthanide chelates and derivatives of these compounds. One criteria in the choice of appropriate fluorescent compounds is that each compound in a set of fluorescent compounds used to analyze a mixture is selected to have a fluorescence lifetime, under relevant experimental conditions, which is distinguishable from the fluorescence lifetimes of some or all of the other compounds in the set. Other appropriate fluorophores and combinations of fluorophores will be apparent to those of skill in the art.

The primary consideration in the choice of appropriate fluorescent compounds is selecting each compound in a set of fluorescent compounds used to analyze a mixture to have a fluorescence lifetime which, under relevant experimental conditions, is distinguishable from the fluorescence lifetimes of the other compounds in the set. Other appropriate fluorophores and combinations of fluorophores will be apparent to those of skill in the art.

In a presently preferred embodiment, the fluorophore is derivatized with a reactive functionality through which the fluorophore is tethered to a component of the mixture that is being analyzed. Many reactive fluorescent molecules are known by and readily available to those of skill in the art. Appropriate reactive fluorescent derivatives are commercially available (*e.g.*, Molecular Probes Inc., Eugene, Oregon) or they can be synthesized by means well known in the art.

Fluorescent agents which are reactive towards amines (e.g., isothiocyanates, carboxylic acids, succinimidyl esters, sulfonyl halides, dialdehydes), thiols (e.g., iodoacetamides, maleimides, alkyl halides, aziridines, epoxides, disulfides), alcohols (isocyanates, acylnitriles, acid chlorides), aldehydes, ketones, vicinal diols (hydrazine derivatives, amines) and carboxylic acids (amines, alkyl halides, trifluoromethansulfonates) are preferred for use in the present invention.

Before using the conjugates in the present invention, models of the conjugates are preferably characterized as to spectral characteristics including optimal excitation and emission wavelengths and fluorescence lifetimes. All of these properties of the conjugates can be determined using standard techniques. The fluorescence lifetime of the conjugate may be dependent on the fluorophore to analyte ratio in the sample. The optimal ratio between the fluorophore and the analyte can be determined experimentally.

In choosing two or more fluorophores for use in the present invention, the following criteria generally pertain:

a. Ideally, the fluorophores should have substantial overlap of absorption wavelengths so that they can all be efficiently excited at a single wavelength;
b. The emission wavelengths should have substantial overlap of emission bands so that the fluorescence contribution of each label can be monitored at a single wavelength; and
c. The differences in the fluorescence lifetimes between fluorophores is typically at least about 5 nanoseconds.

The use of a set of fluorescent compounds with overlapping emission bands allows the excitation of all of the compounds of the set to occur in a substantially simultaneous manner. In contrast, when the compounds are distinguished on the basis of the positions of their absorption bands, each member of the set of compounds must have a unique absorption band and each compound must be excited at a different wavelength.

When more than one fluorescent compound is used, a set of compounds which have substantially similar emission maxima are preferred. The use of a set of compounds having this characteristic allows the compounds to be detected and identified by monitoring their emission at one wavelength, or within a narrow range of wavelengths.

In order to detect those compounds which are excited by, or which emit, electromagnetic energy at similar wavelengths, the compounds will preferably have lifetimes which are sufficiently different to allow them to be clearly distinguished. Thus, a useful set of compounds will include a group of compounds whose lifetimes differ from each other by at least 5 nanoseconds.

In view of the availability of an array of appropriate compounds, it is well within the capabilities of one skilled in the art to choose a reactive fluorescent molecule or set of molecules that is appropriate to the practice of the present invention. A broad range of appropriate fluorophores are commercially available from sources such as Molecular Probes Inc. (Eugene, Oregon).

Measurement of Fluorescence Lifetimes

Two techniques for measuring fluorescence lifetimes of fluorophores are the pulse decay method and the phase-modulation method. In the pulse decay method, the sample is excited with a pulse of light, or a series of pulses, and the time-dependent decay of the fluorescent emission is measured. In the phase-modulation method, the sample is excited with light having a time-dependent intensity, e.g., sinusoidally modulated light, and the time-dependent fluorescence emission is detected. The phase shift of the fluorescence emission relative to the exciting light is used to calculate the fluorescence lifetime. Thus, in presently preferred embodiments, the detecting is provided by a pulse method or a phase-modulation method.

Figure 2 depicts an example of excitation pulses and of the resulting fluorescence emissions from fluorescent dyes having different fluorescence lifetimes. As shown in Figure 2, the excitation pulses are represented as square waves or pulses. Each excitation pulse is absorbed by the fluorescent dye and the fluorescent dye subsequently fluoresces with emission characteristics as shown depending on whether the particular dye has a long or short fluorescence lifetime. As can be seen, the fluorescent dye having a long fluorescence lifetime exhibits a slower rate of attenuation relative to the fluorescent dye having a short fluorescent lifetime. To determine the fluorescence lifetime, a

measurement is taken of the time it takes for the decaying fluorescent pulse to decay to 1/e, or approximately 37%, of the maximum amplitude.

Figure 3 depicts an example of a sinusoidally modulated excitation signal and of the resulting fluorescent emission signals from fluorescent dyes having different fluorescent lifetimes. As shown in Figure 3, the excitation light is represented as a sinusoidal wave. The excitation light is continually absorbed by the fluorescent dye, and the fluorescent dye subsequently fluoresces with emission characteristics as shown depending on whether the particular dye has a short or a long fluorescence lifetime. The fluorescent dye having a longer fluorescence lifetime exhibits a greater phase shift relative to the excitation signal than does the fluorescent dye having a shorter fluorescence lifetime, as shown by the arrows in Figure 3. As is well known, the fluorescence lifetime can be calculated from either the demodulation factor m or the phase shift ϕ as follows:

$$\tau = (1/\omega) \tan \phi \quad (4)$$

or

$$\tau = (1/\omega)[(1/m^2)-1]^{1/2}, \quad (5)$$

where ω is the angular modulation frequency.

According to these embodiments, fluorescence emission signals that reach the detector are measured as a function of time. Thus, the overall detected signal is a superposition of several signals (e.g., a background signal and one analyte-specific signal; or signals from different analytes in the case of a multiple analyte assay, etc.). The individual contributions to the overall fluorescence signal detected are distinguishable by the different fluorescence lifetimes (decay rates) exhibited by each signal. The amplitude of each component of the overall fluorescence signal is proportional to the species responsible for that component. In order to distinguish different fluorescence lifetimes (fluorescent decay times) in the same signal, the individual fluorescence lifetimes (fluorescent decay times) should be significantly different, which is often the case with the background signal compared to a fluorescent probe.

Because of the ability of the method of the invention to distinguish between fluorophores on the basis of their different lifetimes, the position of the absorption, excitation and emission maxima are less relevant to the present technique than those techniques that make use of these maxima for compound identification, *e.g.*, as

described in U.S. Pat No. 5,171,534, issued to Smith *et al.* Thus, in a preferred embodiment, a first fluorescent label and a second fluorescent label have emission maxima that occur at substantially the same wavelength. In some cases, however, additional resolution and/or complexity of analysis can be accomplished by using labels which have distinguishable excitation and/or emission maxima. Thus, in another presently preferred embodiment, the first fluorescent label and the second fluorescent label have an emission maximum that occurs at a substantially different wavelength.

Detection Systems

In accordance with this invention, individual compounds are identified at a detection station by stimulating and detecting their fluorescence and measuring the lifetime of the detected fluorescence. Useful detection stations will typically include three components: an excitation source, an optical system and a detector. Those of skill in the art will be able to choose from a range of art recognized components and combinations of components to practice the present invention. *See* also, The Photonics Design and Applications Handbook, books 1, 2, 3 and 4, published annually by Laurin Publishing Co., Berkshire Common, P.O. Box 1146, Pittsfield, MA for common sources for optical components. Additional considerations for use of the present invention with microfluidic devices and specific example detection systems are discussed below.

A range of appropriate detection stations are available commercially. In a preferred embodiment, the detection station will use an excitation source which is a laser or a nanosecond flash lamp. Useful lasers include, but are not limited to, argon ion pumped and mode-locked Ti:sapphire lasers which provide tunable femto- or picosecond pulses. Suitable argon and mode-locked Ti:sapphire lasers are available as models INNOVA 420 and MIRA 900, respectively from the Laser Products Division of Coherent, Inc. (Palo Alto, CA). Other suitable lasers include Nd:YAG lasers such as models ANTARES 76-S, 468-ASE, 7950, 701 and 7049 from the Laser Products Division of Coherent, Inc. (Palo Alto, CA).

Nanosecond flash lamps that generate pulses on the nanosecond time-scale are commercially available. One suitable lamp is available from Photon Technology International (Monmouth Junction, N.J.) and generates pulses of 1.6 nanoseconds.

The optical system can be constructed to have any useful configuration known in the art and can comprise any number of lenses, mirrors, prisms, beam splitters and dispersive elements (*e.g.*, monochronomators and diffraction gratings) and the like.

The detector can be any device that is capable of detecting photons including, but not limited to, photodiodes, photocathodes, photomultiplier tubes and the like. A presently preferred detector utilizes a stroboscopic detection system such as that described in James *et al., Rev. Sci. Instrum.* **63**:1710 (1991).

The reactants or components to be detected after labeling with fluorescent labels distinguishable by their decay times can be elements of essentially any assay or reaction which is adaptable to a flowing or electrophoretic format; thus, while often described in terms of sequencing reactions, it will be understood that the reactants or assay components herein can comprise a moiety derived from any of a wide variety of components, including, antibodies, antigens, ligands, receptors, enzymes, enzyme substrates, amino acids, peptides, proteins, nucleosides, nucleotides, nucleic acids, organic molecules, monomers, polymers, drugs, polysaccharides, lipids, liposomes, micelles, toxins, biopolymers, therapeutically active compounds, molecules from biological sources, blood constituents, cells or the like. No attempt is made herein to describe how known assays utilizing these components are currently practiced. A wide variety of microfluidic assays are practiced using these components. *See, e.g.*, WO 98/00231 entitled "HIGH THROUGHPUT SCREENING ASSAY SYSTEMS IN MICROSCALE FLUIDIC DEVICES" by Parce *et al.*

Figure 4 is a block diagram of a fluorescence detection system 200 according to one embodiment of the present invention. Detection system 200 includes an excitation source 210 for exciting fluorophores in a detection region 240 with one or more excitation pulses, and a sensor, or detector, 220 for detecting fluorescence emission signals from detection region 240. Fluorescence detector 220 is coupled to a processor 230 which analyzes signals from fluorescence detector 220 to determine fluorescence lifetimes. A beamsplitter element 250, positioned between excitation source 210 and detection region 240, is optionally provided to allow a substantial portion of the excitation signal incident from excitation source 210 to pass through to detection region 240, and to redirect a substantial portion of the radiation incident from detection region 240, including fluorescence emissions, toward fluorescence detector 220.

In preferred aspects, excitation source 210 is a radiation source that can be turned on and off very rapidly or which can be modulated rapidly at a rate up to many million times per second, either directly or by using an opto-mechanical device. According to preferred aspects, excitation source 210 emits radiation having a wavelength

in the range of about 350 nm to about 1000 nm, and which is modulated with a reference signal having a frequency in the range of about 1MHz to about 100MHz. More preferably, excitation source 210 is a laser diode that emits visible radiation having a wavelength of approximately 635nm, and which is modulated at approximately 10MHz with pulses having a width of approximately 10ns. One suitable laser diode is Hitachi's Laser Diode Model HL6320G. Pulse generator 260 is provided according to this embodiment to pulse the excitation source at the desired frequency to obtain the desired modulation and pulse width characteristics. Preferably, the time between pulses is equal to or greater than about five times the decay time of each fluorophore. One of skill in the art will, of course, be able to determine other suitable modulation frequencies and characteristics without undue experimentation depending on the particular characteristics of the fluorophores being analyzed.

Excitation source 210 according to alternate embodiments includes other suitable excitation sources such as a laser, a flash-lamp, a light emitting diode (LED), or any other controllable radiation source that emits radiation at the desired wavelength(s). Useful lasers suitable for use with the present invention include, but are not limited to, argon ion pumped lasers and mode-locked Ti:sapphire lasers that provide tunable mili-second, nano-second, pico-second or femto-second pulses. Suitable mode-locked Ti:sapphire lasers and ND:YAG lasers are available as models MIRA 900 and INFINITY, respectively from the Laser Products Division of Coherent, Inc. (Palo Alto, CA). Other suitable lasers include Nd:YAG lasers such as models ANTARES 76-S, 468-ASE, 7950, 701 and 7049 from the Laser Products Division of Coherent, Inc. (Palo Alto, CA). Flash-lamps that generate nanosecond pulses are commercially available. One suitable lamp is available from Photon Technology International (Monmouth Junction, N.J.) and generates 1.6 nanoseconds pulses.

Pulse generator 260 is optionally provided for use with an excitation source requiring modulation, e.g., for pulsing a laser diode, flash-lamp or arclamp, for turning a laser, such as an excimer or HeNe laser, on and off, or for pulsing a flash-lamp-pumped laser. An electro-optical chopping device (not shown), located between excitation source 210 and detection region 240, can also be used to physically chop a continuous excitation signal into a series of pulses as is well known. Other techniques for generating excitation pulses, such as, for example, mode locking and Q-switching, will be readily apparent to those of skill in the art.

As noted above, beamsplitter element 250 is optionally provided in one embodiment to pass excitation light to detection region 240 and to reflect light, including fluorescent emissions from detection region 240 toward fluorescence detector 220. More specifically, beamsplitter element 250 is preferably selected so as to allow a substantial portion of the radiation emitted from the excitation source having a wavelength below a threshold wavelength to pass to detection region 240, and to reflect toward fluorescence detector 220 a substantial portion of radiation incident from detection region 240 having a wavelength above the threshold wavelength. For example, according to preferred aspects, beamsplitter element 250 includes a planar wavelength-sensitive beamsplitter that allows substantially all incident light having a wavelength below about 650nm to pass, and reflects substantially all incident light having a wavelength above about 650nm. As shown in Figure 4, in one embodiment the planar beamsplitter is situated at a 45 angle relative to the incident light, but the beamsplitter may be situated at any desirable angle depending on the beamsplitter's specific properties and the relative position of detector 220.. Alternatively, beamsplitter element 250 can include a prism beamsplitter or a diffraction grating selected and appropriately positioned to reflect and transmit appropriate wavelengths depending on the selected excitation source. Additionally, beamsplitter element 250 can include a beamsplitter that is polarization-dependent.

In one embodiment, fluorescence detector 220 includes a photo multiplier tube (PMT) that measures fast light signals with low intensity and outputs a corresponding proportional signal to processor 230. Fluorescence detector 220 must be fast enough to convert the fluorescence emission signal into a proportional electrical signal. Therefore, fluorescence detector 220 must operate at a rate faster than the decay times of the particular fluorophores being distinguished. Alternatively, fluorescence detector 220 can include an avalanche photo diode or a photodiode, or any other light detection device that measures fast light signals at low intensity and outputs a proportional signal to processor 230. Additionally, as PMTs tend to decrease in efficiency as the wavelength of detected light increases, in some embodiments where fluorescence emissions in the red to infrared wavelengths are to be detected the use of an avalanche photodiode is preferred.

In operation, excitation pulses from excitation source 210 irradiate detection region 240 and excite fluorophores therein, thereby causing the fluorophores to fluoresce. Fluorescence detector 220 detects the resulting fluorescence emissions, either

directly or by reflection from beamsplitter element 250, and generates a proportional signal. Processor 230 receives and analyzes the signal from fluorescence detector 220, which is proportional to the overall fluorescence emissions signal received by fluorescence detector 220 from detection region 240. In one embodiment, processor 230 is coupled to pulse generator 260. In this embodiment, processor 230 receives a reference signal from generator 260, which is proportional to the reference signal used to modulate excitation source 210. Processor 230 uses this reference signal as a reference for determining the fluorescence lifetimes from the signal received from fluorescence detector 220. According to one embodiment, it is not necessary that processor 230 be able to quantify each fluorophore, but, rather that it is able to categorize and distinguish each fluorophore effectively. For example, when used to sequence a nucleic acid in a prepared nucleic acid sample that is separated in an electrophoretic gel transported across detection region 240, processor 230 determines the nucleotide sequence by the relative characteristics of the fluorescent decay times of the different fluorescent labels or probes used.

Figure 5 is a block diagram of a fluorescence detection system 300 according to an alternate embodiment of the present invention. Detection system 300 includes an excitation source 310 for exciting fluorophores in a detection region 340 with an excitation signal having a time-dependent intensity, e.g., sinusoidally modulated light, and a detector 320 for detecting fluorescence emission signals from detection region 340. Fluorescence detector 320 is coupled to a processor 330 which analyzes signals from fluorescence detector 320 to determine fluorescence lifetimes. A beamsplitter element 350, positioned between excitation source 310 and detection region 340, is optionally provided as discussed above to allow a substantial portion of the excitation signal incident from excitation source 310 to pass through to detection region 340, and to redirect a substantial portion of the radiation incident from detection region 340, including fluorescence emissions, toward fluorescence detector 320.

In preferred aspects, excitation source 310 is a radiation source that emits sinusoidally modulated radiation or which can be continuously modulated to emit sinusoidally varying radiation. According to preferred aspects, excitation source 310 emits radiation having a wavelength in the range of about 350 nm to about 1000 nm, and which is modulated with a reference signal having a frequency in the range of about 1MHz to about 100MHz. More preferably, excitation source 310 is a laser diode that

emits visible radiation having a wavelength of approximately 635nm, and which is modulated at approximately 10MHz. One suitable laser diode includes Hitachi's Model HL6320G. Oscillator 360 is provided according to this embodiment to modulate the excitation source at the desired frequency and amplitude to obtain the desired excitation signal characteristics. One of skill in the art will, of course, be able to determine other suitable modulation frequencies without undue experimentation depending on the particular characteristics of the fluorophores being analyzed.

Other suitable excitation sources include any radiation source that emits, or which can be controlled to emit, radiation having a time-dependent intensity, such as a laser, a flash-lamp, a light emitting diode (LED), or the like.

Fluorescence detector 320, in one embodiment, includes a photomultiplier tube (PMT) that measures fast light signals with low intensity and outputs a corresponding proportional signal to processor 330. Alternatively, fluorescence detector 320 can include an avalanche photodiode or a photodiode, or any other light detection device that measures fast light signals at low intensity and outputs a proportional signal to processor 330.

In one embodiment of the present invention, processor 320 includes, or is coupled to, a phase-locked loop (not shown). As is well known, a phase-locked loop (PLL) is useful for determining the phase difference and demodulation factor between two signals. In the present invention, the PLL compares a reference signal received from oscillator 360 with the signals received from the fluorescence detector 320 to determine the differences in phase between the signals. Processor 330 then determines the fluorescence lifetimes of the corresponding fluorophores based on the phase difference or demodulation factor determined by the PLL.

In operation, oscillator 360 generates a reference signal at the desired frequency. Excitation source emits modulated excitation signals in response to the reference signal received from oscillator 360. The excitation signals from excitation source 310 irradiate detection region 340 and excite fluorophores therein, thereby causing the fluorophores to fluoresce. Fluorescence detector 320 detects the resulting fluorescence emissions, either directly or by reflection from beamsplitter element 350, and generates a proportional signal. Processor 330 receives and analyzes the signal from fluorescence detector 320, which is proportional to the overall fluorescence emissions signal received by fluorescence detector 320 from detection region 340. In one

embodiment, processor 330 receives a reference signal from oscillator 360, which is proportional to the reference signal used to modulate excitation source 310. Alternatively, processor 330 receives a reference signal from excitation source 310. Processor 330 uses the received reference signal as a reference for determining the fluorescence lifetimes from the signal received from fluorescence detector 320. For example, in one embodiment, a PLL compares the reference signal with the signals from fluorescence detector 320 to determine the phase differences. Processor 330 then determines the lifetimes of the fluorophores being studied. However, it is not necessary that processor 330 be able to quantify each fluorophore, but, rather that it is able to categorize and distinguish each fluorophore effectively. For example, when used to sequence a nucleic acid in a prepared nucleic acid sample that is separated in an electrophoretic gel transported across detection region 340, processor 330 determines the nucleotide sequence by the relative characteristics of the fluorescent lifetimes of the different fluorescent labels or probes used.

Figure 6 is a block diagram of a fluorescence detection system 400 according to another alternate embodiment of the present invention. Detection system 400 includes an excitation source 410 for exciting fluorophores in a detection region 440 with an excitation signal, and a detector 420 for detecting fluorescence emission signals from detection region 440. Fluorescence detector 420 is coupled to a processor 430 which analyzes signals from fluorescence detector 420 to determine fluorescence lifetimes. A beamsplitter element 450, positioned between excitation source 410 and detection region 440, is optionally provided as discussed above to allow a substantial portion of the excitation signal incident from excitation source 410 to pass through to detection region 440, and to redirect a substantial portion of the radiation incident from detection region 440, including fluorescence emissions, toward fluorescence detector 420. Modulator 460 is provided to modulate excitation source 410 to obtain the desired excitation signal characteristics. For example, in one embodiment similar to Figure 5, modulator 460 includes an oscillator that generates a reference signal having a desired frequency and amplitude. In this embodiment, excitation source emits radiation having a time-dependent intensity, e.g., sinusoidally modulated light, in response to the reference signal. In another embodiment similar to Figure 4, modulator 460 includes a pulse generator that pulses excitation source 410.

Fluorescence detection system 400 also includes additional optical elements for enhancing the excitation and detection capabilities of system 400. Optical elements 470 and 472, positioned between excitation source 410 and detection region 440, are optionally provided according to one embodiment to assist in directing and focusing the excitation signal onto detection region 440. Optical elements 470 and 472 can include focusing lenses, mirrors, or any other optical elements as are well known and which are useful for collimating, directing and focusing radiation depending on the desired system layout and characteristics. Optical element 474 is optionally provided in one embodiment to assist in directing and focusing the fluorescence emissions signals from detection region 440 onto fluorescence detector 420. Optical element 474 in one embodiment includes a focusing lens selected accordingly depending on whether fluorescence emission signals are received by fluorescence detector 420 directly from detection region 440 or via reflection from beamsplitter element 450.

Filter elements 480 and 482 are optionally provided to avoid an overlap of the excitation source spectra and the detectable fluorescence emission spectra. Additionally, filter element 480 can be used to prevent undesirable wavelengths, other than the desired excitation wavelength, that may also be emitted by excitation source 410 from irradiating detection region 440. Filter element 482 can be used to filter unwanted background noise (light) and fluorescence emissions from certain solid support materials in the detection region, e.g., microchannel capillary tubes and the like. An electronic filter can also be used to filter out background noise and unwanted fluorescence emissions from the fluorescence signal received by fluorescence detector 420. In one embodiment, the signal from detector 420 is electronically filtered so that only the emitting frequency (e.g., 10MHz) is detected. The resulting signal can then be compared to the modulation reference signal to determine the fluorescence lifetimes or to determine which is the dominating fluorescence lifetime.

B. Sequencing Techniques

Definitions

As used herein, the term "DNA" or "deoxyribonucleic acid" shall be construed as collectively including DNA containing classical nucleotides, DNA containing one or more modified nucleotides (*i.e.*, fluorescently tagged nucleotides containing a chemically modified base, sugar and/or phosphate), DNA containing one or more nucleotide analogs, and combinations of the above).

As used herein, the term "nucleotide" shall be construed as collectively including all of the forms of nucleotides described *supra* in addition to RNA and derivatives of RNA analogous to those of DNA discussed above.

As used herein the term "polymer" refers to molecules having two or more subunits (*e.g.*, dinucleotides).

As used herein, the term "nucleic acid" is used interchangeably with RNA and DNA and this term can refer to monomeric, oligomeric or polymeric species of these molecules.

The methods and devices of the invention can also be utilized to sequence polymeric and oligomeric molecules including, but not limited to, DNA, RNA, peptides, polysaccharides and the like. In the interest of brevity, the discussion that follows focuses on techniques for sequencing nucleic acids. One of skill in the art will appreciate that with readily practiced modifications, the methods and apparatus of the invention can be utilized to sequence other polymeric molecules such as peptides, proteins, polysaccharides and the like.

Thus, in a second aspect, the present invention provides a method of sequencing a nucleic acid polymer of interest. In this aspect of the invention, the method comprises performing a sequencing reaction on the nucleic acid polymer to produce a nested set of sequence fragments. Any of the sequencing reactions known in the art is appropriate for use in this aspect. Thus, methods which chemically or enzymatically degrade or synthesize nucleic acids are of use in practicing the present invention. *See*, for example Maxam and Gilbert, *Proc. Natl. Acad. Sci. USA* **74**: 560 (1977). When methods which synthesize nucleic acid polymers are utilized, an embodiment, involves producing a plurality of nucleic acid polymers complementary to a region of the nucleic acid polymer of interest. See, for example, Sanger *et al., Proc. Natl. Acad. Sci. USA* **74**: 5463 (1977).

During the course of the sequencing reaction, one or more fluorescent labels is incorporated into either the nucleic acid being sequenced or a sequence complementary to the nucleic acid being sequenced. Several methods for performing this incorporation are known in the art. A non-limiting list includes the Sanger, Sanger dideoxy and Maxam-Gilbert sequencing methodologies. These methods are discussed in greater detail below.

Sequencing reaction mixtures that are useful in practicing the present invention include those that contain the nucleic acid to be sequenced and a fluorescent label. The fluorescent label is attached to a first labeled nucleic acid selected from the group consisting of labeled nucleic acids, labeled nucleic acid polymers and combinations thereof. As discussed above, the fluorescent label will, following excitation, emit electromagnetic energy that is characterized by a distinct and detectable lifetime. When more than one fluorescent label is utilized in the sequencing reaction mixture, each of the labels will have distinct and detectable fluorescence lifetimes.

Each of the above-enumerated sequencing methodologies can be used to practice the present invention. Those of skill in the art have ready access to a body of techniques for forming appropriate sequencing reaction mixtures for use in each of these methods. In a preferred embodiment, the sequencing reaction mixture further comprises a second labeled nucleic acid which is a member selected from the group consisting of labeled nucleic monomers and labeled nucleic acid polymers, wherein said nucleic acid bears a second fluorescent label. The second fluorescent label has a fluorescence emission that has a characteristic fluorescence lifetime.

In this embodiment, the second labeled nucleic acid can be a polymeric species such as an oligonucleotide (*e.g.*, a primer or a dimer, trimer, *etc.*). When the nucleic acid is polymeric, the component bases of the polymer can be identical or they can be different over the length of the strand. Useful monomers include, for example, nucleotides, deoxynucleotides, dideoxynucleotides and modified derivatives thereof.

The second labeled nucleic acid can be an oligonucleotide primer that is used to start nucleic acid synthesis at a second region of the nucleic acid being sequenced. Alternatively, the second nucleic acid can also be a dideoxynucleotide such that chain elongation is terminated upon the dideoxynucleotide's incorporation into a growing nucleic acid.

In still further preferred embodiments, the sequencing reaction mixture further comprises additional labeled nucleic acids. The labels on these additional labeled nucleic acids will also have a fluorescence emission that has a characteristic fluorescence lifetime. It will be clear to one of skill in the art that any number of labeled nucleic acids can be used in a sequencing reaction mixture.

Thus, in certain nucleic acid sequencing procedures, more than four distinct nucleic acids will be present. In these embodiments, the first labeled nucleic acid

bearing a first fluorescent label is a member of a plurality of unique labeled nucleic acid species. Similar to the above-described embodiments, the fluorescent label has a fluorescent emission that has a characteristic fluorescence lifetime.

Moreover, the set of labeled nucleic acids can include a mixture of nucleic acid species. For example, a sequencing reaction mixture can include a primer or a primer and one or more labeled dideoxynucleotides. Alternatively, another exemplary sequencing reaction mixture can include one or more labeled dideoxynucleotides and one or more deoxynucleotides with or without a primer present in the mixture. Other useful sequencing reaction mixture compositions will be apparent and readily accessible to those of skill in the art.

The method of the invention can be carried out by combining all of the labeled species in a "one pot" reaction or, alternatively, one or more of the labeled species can be segregated into one or more reaction vessels. In a presently preferred embodiment, the sequencing reaction is carried out with all of the fluorescently labeled species together as a mixture in a "one pot" reaction.

In another preferred embodiment, the sequencing reaction is performed following the Sanger procedure. *See*, for example, Sanger *et al., Proc. Natl. Acad. Sci. USA* **74**: 5463 (1977). In this method, each of the labeled nucleic acids bearing a different fluorescent tag is incorporated into a polymeric nucleic acid. This embodiment can utilize a "one pot" reaction or, alternatively, one or more labeled species can be segregated and reacted in a separate reaction vessel. The labeled nucleic acids can be labeled primers, labeled deoxynucleotides, labeled dideoxynucleotides or combinations thereof.

In the embodiments which do not utilize a "one pot" reaction scheme, the method further comprises a second sequencing reaction mixture comprising the nucleic acid polymer of interest and a second labeled nucleic acid bearing a second fluorescent label, wherein the second fluorescent label has a fluorescence emission, the emission having a characteristic fluorescence lifetime.

When the nucleic acid to be sequenced contains more than two bases, additional sequencing mixtures can be optionally utilized. Thus, in yet another preferred embodiment, the method of the invention further comprises a third sequencing reaction mixture. Similar to the other sequencing reaction mixtures, the third sequencing mixture comprises the nucleic acid polymer of interest. The third sequencing reaction mixture

also comprises a third labeled nucleic acid which is labeled with a third fluorescent label, wherein the third fluorescent label has a fluorescence emission which has a characteristic fluorescence lifetime.

In another preferred embodiment, the method of the invention further comprises a fourth sequencing reaction mixture that, similar to the mixtures discussed above, comprises a fourth labeled nucleic acid.

When two or more unique nucleic acid bases are present in a nucleic acid strand, in one embodiment, the present invention utilizes as many sequencing reaction mixtures as there are unique nucleic acid bases. In this embodiment, a particular sequencing reaction mixture is a member of a plurality of unique sequencing reaction mixtures. Each reaction mixture comprises the nucleic acid polymer of interest and a unique labeled nucleic acid bearing a unique fluorescent label, wherein the unique fluorescent label has a fluorescence emission. The emission has a characteristic fluorescence lifetime. The fluorescence lifetime is different for each unique fluorescent label.

In addition to the nucleic acid being sequenced, it will often be desirable or necessary to have one or more additional components in the sequencing mixture. The components can be chosen from a wide range of known enzymes, nucleic acids (*e.g.*, labeled nucleic acids, labeled nucleic acid analogs, fluorescent labeled nucleic acids, oligonucleotides, *etc.*), solvents, buffers, catalysts, acids, bases, surfactants, chelating agents, metal ions and the like. Thus, in a presently preferred embodiment, the sequencing reaction mixture further comprises one or more members selected from the group consisting of polymerases, exonucleases, endonucleases, deoxynucleotides, deoxynucleotide diphosphates, deoxynucleotide triphosphates, dideoxynucleotides, dideoxynucleotide diphosphates, dideoxynucleotide triphosphates, nucleotide analogs and nucleoside analogs and combinations thereof.

When the method of the invention is used to sequence nucleic acids and the sequencing reaction mixture contains fluorescent labels and nucleic acids, it is preferred that the nucleic acids labeled with the fluorescent labels and that the nucleic acids are members selected from the group consisting of nucleotides, nucleosides, nucleoside diphosphates, nucleoside triphosphates, dideoxynucleosides, deoxynucleotides, deoxynucleoside diphosphates, deoxynucleoside triphosphates, dideoxynucleosides, dideoxynucleotides, dideoxynucleoside diphosphates,

dideoxynucleoside triphosphates, nucleotide analogs and nucleoside analogs and combinations thereof.

Both natural and "unnatural" nucleotides can be derivatized with fluorescent labels and used to practice the present invention. Thus, in certain preferred embodiments, the nucleotide bearing a fluorescent label is a non-natural nucleotide.

In yet another preferred embodiment, the invention provides a sequencing reaction mixture as described above. In a still further preferred embodiment, the invention provides a kit comprising one or more sequencing mixtures as described above.

A number of nucleic acid sequencing techniques can be used in conjunction with the present invention. Broad classes of suitable sequencing techniques include those that use a chemical or enzymatic degradation process and those that use enzymatic synthesis of nucleic acids.

Methods that utilize chemical or enzymatic degradation of nucleic acids are known within the art and are suitable for use in practicing the present invention. *See*, for example, Ansorge *et al.*, *Nucleic Acids Res.* **16**:2203-2206 (1988); Porter *et al. Nucleic Acids Research* **25**:1611-1617 (1997). Additionally, methods that utilize the enzymatic synthesis of nucleic acids can be used to practice the present invention including, for example, the Sanger method and its modifications. These methods are discussed in greater detail below.

Enzymes used in DNA Sequencing

Two classes of enzyme activity that have been employed in certain methods used to sequence DNA are DNA polymerase and exonuclease activity. A DNA polymerase is an enzyme that has the ability to catalytically synthesize new strands of DNA *in vitro*. The DNA polymerase carries out this synthesis by moving along a preexisting single DNA strand ("the template") and creating a new strand complementary to the existing strand by incorporating single nucleotides one at a time into the new strand following the base-pairing rule.

In contrast to polymerase activity, exonuclease activity refers to the ability of an enzyme (an exonuclease) to cleave off a nucleotide at the end of a DNA strand. Enzymes are known which can cleave successive nucleotides off a single DNA strand, working from the 5' end of the strand to the 3' end; such enzymes are termed single-stranded 5' to 3' exonucleases. Other enzymes are known which perform this operation

in the opposite direction (single-stranded 3' to 5' exonucleases). There also exist enzymes that can cleave successive nucleotides from the end of a single strand of a double-stranded DNA molecule. These enzymes are termed double-stranded 5' to 3' or 3' to 5' exonucleases, depending on the direction in which they proceed along the strand. Exonucleases are characterized as being distributive or processive in their action. Distributive exonucleases dissociate from the DNA following each internucleotide bond cleavage, whereas processive exonucleases will hydrolyze many internucleotide bonds without dissociating from the DNA.

Thermostable polymerases (*e.g.*, Taq) are also useful in performing the polymerase chain reaction in conjunction with the sequencing method of the invention. See, for example, U.S. Patent No. 4,683,202; Arnheim and Levinson, *C&EN* 36-47 (October 1, 1990), Kwoh *et al., Proc. Nat'l. Acad. Sci. USA* **86**:1173 (1989).

Thus, in yet another preferred embodiment, the method of the invention further comprises the use of the polymerase chain reaction to amplify the DNA being sequenced.

Sequencing Ladder Methods

Techniques for sequencing DNA generate fragments of labeled DNA, the lengths of which are sequence dependent, and separate the fragments according to their lengths, for example, by electric field induced migration in a gel or capillary. Such a pattern of sequence-dependent fragment lengths is known as a sequencing ladder.

When a nucleic acid is sequenced, by any of the above-discussed methods, the sequencing mixture will generally be submitted to a separation protocol that separates different populations of oligonucleotides on the basis of their size, charge, hydrophobicity and combinations of these properties. Thus, in a preferred embodiment, when an additive method such as the Sanger method is used, the method of the invention further comprises separating the complementary nucleic acid polymers into distinct populations, each of the populations consisting of nucleic acid polymers of about the same size.

Although any appropriate separation methodology can be utilized including, electrophoresis (gel, capillary, *etc.*), chromatography (HPLC, size exclusion, affinity, *etc.*), precipitation, and the like, in a presently preferred embodiment, the separating is provided by a method selected from the group consisting of electrophoresis, electroosmosis, electrokinesis, chromatography and combinations thereof.

The fragments of a sequencing ladder can be generated by either: (a) cleaving the DNA in a base-specific manner, or (b) synthesizing a copy of the DNA wherein the synthesized strand terminates in a base-specific manner.

The Maxam-Gilbert technique for sequencing involves the specific chemical cleavage of DNA. According to this technique, four samples of the same DNA are each subjected to a different chemical reaction to effect preferential cleavage of the DNA molecule at one or two nucleotides of a specific base identity. By adjusting the conditions to obtain only partial cleavage, DNA fragments are thus generated in each sample whose lengths are dependent upon the position within the DNA base sequence. Thus, after partial cleavage, each sample contains DNA fragments of different lengths each of which ends in the same one or two of the four nucleotides. *See*, Maxam and Gilbert, *Proc. Natl. Acad. Sci. USA* **74**:560 (1977)

The plus/minus DNA sequencing method involves: (a) use of polymerases to generate complementary labeled DNA oligonucleotides of different lengths; (b) (the "minus" system) in four separate reaction vessels, reaction of one half of the generated DNA with DNA polymerase and three out of the four nucleotide precursors; (c) (the "plus system") in four separate reaction vessels, reaction of the remaining half of the generated DNA with DNA polymerase and only one of each of the four nucleotide precursors. Each reaction mixture generated in steps (b) and (c) is subjected to a separation procedure and the generated fragments are separated from each other by migration. *See*, Sanger and Coulson, *J. Mol. Biol.* **94**:441-448 (1975).

The dideoxy method relies on the enzymatic activity of a DNA polymerase to synthesize DNA fragments with lengths that are sequence dependent. *See*, Sanger *et al., Proc. Natl. Acad. Sci. USA* **74**:5463 (1977). The Sanger dideoxy method utilizes an enzymatically active fragment of the DNA polymerase termed E. coli DNA polymerase I, to carry out the enzymatic synthesis of new DNA strands. The newly synthesized DNA strands include fragments of sequence-dependent length, generated through the use of inhibitors of DNA polymerase which cause the base-specific termination of synthesis. Such inhibitors are dideoxynucleotides that, upon their incorporation by the DNA polymerase, destroy the ability of the enzyme to further elongate the DNA chain due to the dideoxynucleotides' lack of a suitable 3'–OH necessary in the elongation reaction. When a dideoxynucleotide whose base can appropriately hydrogen bond with the template DNA is thus incorporated into the DNA, synthesis of the growing polymer chain

stops. Thus, DNA fragments are generated by the DNA polymerase, the lengths of which are dependent upon the position within the DNA base sequence of the nucleotide whose base identity is the same as the incorporated dideoxynucleotide. The fragments are then submitted to a separation procedure. For a simple introduction to dideoxy sequencing, see, *Current Protocols in Molecular Biology*, F.M. Ausubel *et al.*, eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (Supplement 37, current through 1997) (Ausubel), Chapter 7. Four color sequencing is described in U.S. Pat. No. 5,171,534. Thousands of laboratories employ dideoxynucleotide chain termination techniques. Commercial kits containing the reagents most typically used for these methods of DNA sequencing are available and widely used.

Useful modifications to the Sanger method include, for example, the use of modified T7 DNA polymerase in two-step synthesis reactions as described in U.S. Pat. No. 4,994,372 to Tabor and Richardson and thermal stable DNA polymerases in two-step synthesis reactions as described in U.S. Pat. No. 5,075,216 to Brow *et al.* and thermal cycle DNA sequencing (Craxton, *Methods* **3**:73-74 (1991)), all of which are incorporated herein by reference. The two-step labeling protocol and thermal cycle protocols employing thermostable DNA polymerases can be used to minimize problems associated with DNA template secondary structure.

The recent advent of thermal cycle sequencing methodologies has increased the number of suitable sequencing templates such as lambda and cosmid templates, polymerase chain reaction (PCR) products and direct plasmid sequencing from bacterial colonies. Because the thermal cycle methods employ heat denaturation, the requirement for alkaline denaturation and ethanol precipitation of double-stranded templates has been eliminated.

Current methods, prospects for automation and novel methods of DNA sequencing are reviewed by Martin and Davies (*Bio/Technology* **4**:890-895 (1986)), Bains (*Bio/Technology* **8**:1251-1256 (1990) and Hunkapiller *et al.* (*Science* **254**:59 (1991)), which are incorporated herein by reference.

In addition to the Sanger methods of chain termination, new PCR exonuclease digestion methods have also been proposed for DNA sequencing. Direct sequencing of PCR generated amplicons by selectively incorporating boronated nuclease resistant nucleotides into the amplicons during PCR and digestion of the amplicons with a nuclease to produce sized template fragments has been proposed (Porter *et al. Nucleic*

Acids Research **25**:1611-1617 (1997)). In the methods, 4 PCR reactions on a template are performed, in each of which one of the nucleotide triphosphates in the PCR reaction mixture is partially substituted with a 2'deoxynucleoside 5'-[P-borano]-triphosphate. The boronated nucleotide is stocastically incorporated into PCR products at varying positions along the PCR amplicon in a nested set of PCR fragments of the template. An exonuclease which is blocked by incorporated boronated nucleotides is used to cleave the PCR amplicons. The cleaved amplicons are then separated by size using polyacrylamide gel electrophoresis, providing the sequence of the amplicon. An advantage of this method is that it requires fewer biochemical manipulations than performing standard Sanger-style sequencing of PCR amplicons.

Sequencing methods which reduce the number of steps necessary for template preparation and primer selection have been developed and can be applied to the present invention. One proposed variation on sequencing technology involves the use of modular primers for use in PCR and DNA sequencing. For example, Ulanovsky and co-workers have described the mechanism of the modular primer effect (Beskin *et al.*, *Nucleic Acids Research* **23**:2881-2885 (1995)) in which short primers of 5-6 nucleotides can specifically prime a template-dependent polymerase enzyme for template dependent nucleic acid synthesis. A modified version of the use of the modular primer strategy, in which small nucleotide primers are specifically elongated for use in PCR to amplify and sequence template nucleic acids has also been described. The procedure is referred to as DNA sequencing using differential extension with nucleotide subsets (DENS). *See,* Raja *et al.*, *Nucleic Acids Research* **25**:800-805 (1997).

In addition to enzymatic and other chain termination sequencing methods, sequencing by hybridization to complementary oligonucleotides has been proposed, *e.g.*, in U.S. Patent No. 5,202,231, to Drmanac *et al.* and, *e.g.*, in Drmanac *et al. Genomics* **4**:114-128 (1989). Chemical degradation sequencing methods are also well known and still in use; *see,* Maxam and Gilbert, *Methods in Enzymology* **65**:499-560 (1980).

C. SOURCES OF NUCLEIC ACIDS

Nucleic acids to serve as sequencing templates are optionally derived from a natural source or they can be synthetic or recombinant. For example, DNAs can be recombinant DNAs, *e.g.*, plasmids, viruses or the like. A wide variety of molecular and biochemical methods are available for making coding DNAs. Examples of appropriate molecular techniques for generating recombinant nucleic acids, and instructions sufficient

to direct persons of skill through many cloning exercises are found in Berger and Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology* volume 152 Academic Press, Inc., San Diego, CA (Berger); as well as in Sambrook, and Ausubel (both *supra).* Product information from manufacturers of biological reagents and experimental equipment also provide information useful in known biological methods. Such manufacturers include the SIGMA chemical company (Saint Louis, MO), R&D systems (Minneapolis, MN), Pharmacia LKB Biotechnology (Piscataway, NJ), CLONTECH Laboratories, Inc. (Palo Alto, CA), Chem Genes Corp., Aldrich Chemical Company (Milwaukee, WI), Glen Research, Inc., GIBCO BRL Life Technologies, Inc. (Gaithersberg, MD), Fluka Chemica-Biochemika Analytika (Fluka Chemie AG, Buchs, Switzerland), Invitrogen, San Diego, CA, Applied Biosystems (Foster City, CA), Digene Diagnostics, Inc. (Beltsville, MD) as well as many other commercial sources known to one of skill.

Typically, oligonucleotides are used as sequencing primers, or as amplification primers. Most commonly, these DNA or RNA oligonucleotides are made synthetically. Synthetic oligonucleotides are typically synthesized chemically according to common solid phase phosphoramidite triester methods described, *e.g.*, by Beaucage & Caruthers (1981) *Tetrahedron Letts.* 22(20):1859-1862, *e.g.*, using an automated synthesizer, as described in Needham-VanDevanter *et al.* (1984) *Nucleic Acids Res.* 12:6159-6168. Oligonucleotides can also be custom made and ordered from a variety of commercial sources known to persons of skill. In other embodiments, oligonucleotides are made recombinantly according to standard techniques, described, *e.g.*, in Berger, Sambrook and Ausubel, *all supra*.

Oligonucleotides are typically selected to have particular hybridization characteristics with a template DNA to form a duplex with the DNA. The oligonucleotide is typically used as a primer for a processive DNA polymerase in either a sequencing or amplification reaction. Most typically, oligonucleotides are selected to be fully complementary to the selected template DNA, although a portion of the oligonucleotide can be non-complementary (*e.g.*, a portion may act as a labeling or cloning element instead of participating in hybridization, or a single oligonucleotide can be used as a primer for multiple closely related templates in separate assays to reduce individual assay costs). The oligonucleotides are preferably selected to have melting temperatures near the temperature of the reaction, to reduce background hybridization

interactions. It is expected that one of skill is thoroughly familiar with the theory and practice of nucleic acid hybridization and selection of complementary oligonucleotides. *See, e.g.*, Gait (ed.), OLIGONUCLEOTIDE SYNTHESIS: A PRACTICAL APPROACH, IRL Press, Oxford (1984); Kuijpers, *Nucleic Acids Research* 18(17):5197 (1994); Dueholm (1994) *J. Org. Chem.* 59:5767-5773; Agrawal (ed.) METHODS IN MOLECULAR BIOLOGY, volume 20; and Tijssen (1993) LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY-HYBRIDIZATION WITH NUCLEIC ACID PROBES, *e.g.*, part I chapter 2; Overview of principles of hybridization and the strategy of nucleic acid probe assays, Elsevier, New York, provide a basic guide to nucleic acid hybridization. Most typically, oligonucleotide selection steps are performed using simple computer programs, although all of the steps are optionally performed manually. One available computer program for primer selection is the MacVector™ program from Kodak, or the MFOLD program (Genetics Computer Group, Madison WI).

D. FLUORESCENT LABELING OF NUCLEIC ACIDS

Fluorescent tags useful in practicing the present invention can be tethered to any location on a nucleic acid, including sites on the base segment and sites on the sugar segment. Thus, in a preferred embodiment, the fluorescent label is covalently attached to a segment of a nucleic acid which is a member selected from the group consisting of the base segment, the sugar segment and both the base segment and the sugar segment.

In another preferred embodiment, the modified nucleic acid bears at least one fluorescent label and it serves as a primer for nucleic acid synthesis and the method of the invention further comprises annealing the nucleic acid polymer of interest with a primer nucleic acid polymer.

In another preferred embodiment, the fluorescent label is covalently attached to a labeled nucleic acid which is a member selected from the group consisting of the 3'-terminus, the 5'-terminus, an internal position and combinations thereof.

The art is replete with an arsenal of methods for the preparation, purification and characterization of a manifold of derivatized labeled nucleic acids. This subject has recently been reviewed. *See*, Goodchild, *Bioconjug. Chem.* 1:165-187 (1990), which is incorporated herein by reference.

Many of these methods are quite appropriate for use in preparing the various compounds required to practice the present invention. One skilled in the art will be able, without undue experimentation, to choose a suitable method for preparing a desired fluorescently labeled nucleic acid, oligonucleotide or the like. Additionally, as the art of organic synthesis, particularly in the area of nucleic acid chemistry, continues to expand in scope new methods will be developed which are equally as suitable as those now known. The following discussion is offered as representative of the array of compounds and techniques that can be used to modify nucleic acids. Methods useful in conjunction with the present invention, are not to be construed as limited by this discussion.

MODIFICATION OF INTACT OLIGONUCLEOTIDES

A number of techniques have been developed for converting specific constituents of DNA and RNA strands into fluorescent adducts. These techniques have been reviewed. *See*, Leonard and Tolman, in "Chemistry, Biology and Clinical Uses of Nucleoside Analogs," A. Bloch, ed., *Ann. N.Y. Acad. Sci.* **255**:43-58 (1975).

Chemical methods are available to introduce fluorescence into specific nucleic acid bases by the reaction of chloracetaldehyde with adenosine and cytidine to give fluorescent products. The reaction can be controlled with respect to which of the two bases is derivatized by manipulating the pH of the reaction mixture; the reaction at 37 °C proceeds rapidly at the optimum pH of 4.5 for adenosine and 3.5 for cytidine. *See*, *Barrio et al., Biochem. Biophys. Res. Commun.* **46**:597-604 (1972). This reaction is also useful for rendering fluorescent the deoxyribosyl derivatives of these bases. *See*, Kochetkov *et al., Dokl. Akad. Nauk. SSSR C* **213**:1327-1330 (1973).

DNA and RNA can be modified by reacting their cytidine residues with sodium bisulfite to form sulfonate intermediates that are then coupled to reactive nitrogen compounds such as hydrazides or amines. *See*, Viscidi *et al. J. Clin. Microbiol.* **23**:311 (1986) and Draper and Gold, *Biochemistry* **19**:1774 (1980).

RNA can also be labeled at the 3' terminus by selective oxidation. The selective oxidation of the 3' ribose of RNA by periodate yields a dialdehyde which can then be coupled with an amine or hydrazide reagent. Churchich, *Biochim. Biophys. Acta* **75**:274-276 (1963); Hileman *et al. Bioconjug. Chem.* **5**:436-444 (1994).

Fluorescent G derivatives have also been prepared from the natural base upon its reaction with variously substituted malondialdehydes. *See*, Leonard and Tolman,

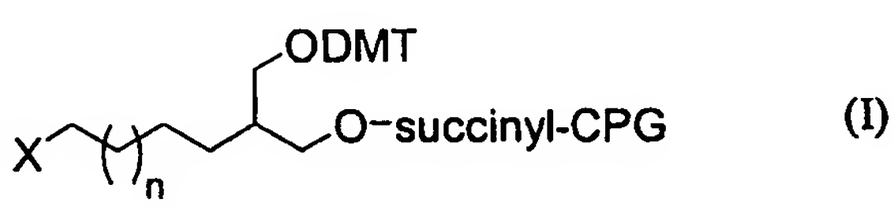
in "Chemistry, Biology and Clinical Uses of Nucleoside Analogs," A. Bloch, ed., *Ann. N.Y. Acad. Sci.* **255**:43-58 (1975).

De Novo Synthesis of Fluorescent Oligonucleotides

In addition to the various methods for converting the bases of an intact oligonucleotide into their fluorescent analogs, there are a number of methods for introducing fluorescence into an oligonucleotide during its *de novo* synthesis.

At least three methods are available for fluorescently tagging a synthetic oligonucleotide. These methods utilize fluorescently tagged supports, fluorescently tagged 5' modification reagents and fluorescently tagged monomers.

The first of these methods utilizes a fluorescently tagged linker that tethers the oligonucleotide strand to the solid support. When the oligonucleotide strand is cleaved from the solid support, the fluorescent tether remains attached to the oligonucleotide. This method affords an oligonucleotide that is fluorescently labeled at its 3'-end. In a variation on this method, the 3'-end of the nucleic acid is labeled with a linker that bears an amine, or other reactive or masked reactive group, which can be coupled to a reactive fluorophore following cleavage of the oligonucleotide from the solid support. This method is particularly useful when the fluorophore is not stable to the cleavage or deprotection conditions. An exemplary derivatized solid support is shown below in Formula I:



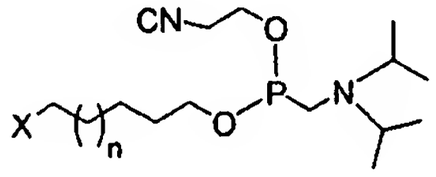
(I)

wherein n is an integer between 1 and 10 and X is a fluorophore or a reactive group such as, for example, NH_2, SH, OH, COOH, or a protected derivative of a reactive group. Methods for protecting these and other reactive groups are known in the art. See, for example, Greene and Wuts, PROTECTIVE GROUPS IN ORGANIC SYNTHESIS, 2nd Ed., John Wiley & Sons, N.Y., 1991.

A second method relies on the selective labeling of the 5' terminus of the oligonucleotide chain. Although many methods are known for labeling the 5' terminus, the most versatile methods make use of phosphoramidites which are derivatized with

fluorophore or, if the fluorophore is unstable under the cleaving and deprotection conditions, a protected reactive functional group. The reactive functional group is labeled with a fluorophore following cleavage and deprotection of the oligonucleotide and deprotection of the reactive functional group.

The 5' derivatizing amidites are coupled to the growing nucleic acid strand as a last synthetic cycle that is generally accomplished in exactly the same manner as the previous steps that incorporated single nucleotides. An exemplary compound useful in this method is displayed below in Formula II:



(II

wherein n is an integer between 1 and 10 and X is a fluorophore or a reactive group such as, for example, NH_2, SH, OH, COOH, or a protected derivative of a reactive group.

Many reagents for effecting these conversions are commercially available from chemical houses such as Glen Research (Sterling, Virginia). Other agents can be prepared *de novo* and the commercial agents can be modified by methods well known in the art.

Modified Labeled Nucleic Acids

Individual nucleotides can be derivatized with fluorescent moieties on the base or sugar components. Modification to the base can occur at exocyclic amines or at the carbons of the ring. *See*, for example, Levina *et al.*, *Bioconjug. Chem*. 4:319-325 (1993). Modification of the sugar moiety can take place at the oxygens of the hydroxyl groups or the carbon atoms of the ribose ring. *See*, for example, Augustyns *et al.*, *Nucleic Acids Symp. Ser*. 24:224 (1991); Yamana *et al.*, *Bioconjug. Chem*. 7:715-720 (1996); Guzaev *et al.*, *Bioconjug. Chem*. 5:501-503 (1994); and Ono *et al.*, *Bioconjug. Chem*. 4:499-508 (1993), and references contained within, the disclosure of each of which is incorporated herein by reference.

The modified labeled nucleic acids can also be 2'-deoxyribonucleic acids which are labeled at the 3'-hydroxyl via, for example, alkylation or acylation. These labeled nucleic acids will function like dideoxynucleic acids, terminating synthesis, when used in the Sanger method.

As discussed above, fluorescent tags useful in practicing the present invention can be tethered to any location on a nucleic acid, including sites on the base segment and sites on the sugar segment. Thus, in a preferred embodiment, the fluorescent label is covalently attached to a segment which is a member selected from the group consisting of the base segment, the sugar segment and both the base segment and the sugar segment.

The methods and devices of the invention can be used to sequence nucleic acids of practically any length. In preferred embodiments, the nucleic acid lengths are within the art established ranges, preferably a size of from about 2 bases to about 100,000 bases, more preferably from about 100 bases to about 10,000 and still more preferably from about 300 bases to about 5000 bases.

The following exemplary embodiment illustrates a method of sequencing a nucleic acid using fluorescent labels and a chemical degradation pathway.

Fully protected oligodeoxyribonucleotides can be prepared on an Applied Biosystems DNA synthesizer using standard β-cyanoethyl phosphoramidite chemistry. *See*, Sinha *et al., Nucleic Acids Res.* **12**:4539-4557 (1984). A portion of the material can be retained for a further synthetic cycle employing (S-trityl-3-mercaptopropyloxy), 2-cyanoethoxy N, N-diisopropylaminophosphine in the condensation step. This phosphoramidite has been synthesized and is known in the art. *See*, Ansorge *et al., Nucleic Acids Res.* **16**:2203-6 (1988).

After removal of the blocking groups and cleavage from the support with ammonia, the S-trityl oligonucleotide can be purified by reverse-phase HPLC. Detritylation with silver nitrate and subsequent reaction of the liberated thiol with 5-iodoacetamidofluorescein can be performed as described in Ansorge *et al., Nucleic Acids Res.* **15**:4593-4602 (1987). The excess dye can be removed by ethanol precipitations of the oligodeoxyribonucleotide. The fluorescein labeled oligodeoxyribonucleotide can be purified by reverse-phase HPLC, prior to sequencing by chemical degradation.

Chemical degradation of oligonucleotides can be performed essentially as described in Rosenthal *et al.*, *Methods Enzymo*l. **155**:301-331 (1987) using Hybond M & G paper (Amersham). Approximately 5 pmol of fluorescein labeled oligomer can be applied to the carrier in 1 μl aliquots. For degradation, the following reagents can be used:

G: with 1% DMS in 50 mM ammonium formate buffer, pH 3.5 for 10 min.;

A + G: with 80% formic acid for 20 min.;

T > Pu: with 0.1mM KMnO4 for 20 min,;

C: with 4M hydroxylamine, pH 6.

After piperidine reaction and lyophilization, the samples can be dissolved in 30% aqueous formamide.

In another exemplary embodiment, a lanthanide chelate serves as the fluorescent label. The chelate is diethylenetriaminepentaacetic acid (DTPA) and it is tethered to the nucleic acid using the corresponding DTPA dianhydride (DTPAA). In this embodiment, the method and device of the invention is used simply to separate and identify, not sequence, different oligonucleotides.

A plasmid, such as plasmid pBR322 is purified and digested according to art-recognized procedures. *See*, Mamatis *et al.* MOLECULAR CLONING: A LABORATORY MANUAL, Cold Springs Harbor Laboratory, Cold Springs Harbor, N.Y., pp. 100-106. In one example digest, the digestion of pBR322 generates 10 fragments with staggered ends ranging from 75 base pairs to 1631 base pairs; the sequence of single-stranded bases at each end is ANT, where N denotes any nucleotide. It is assumed that the exocyclic amines on the exposed bases provide sites for attachment of the DTPA moiety via amide linkages formed between these amines and a carboxylate group of the DTPA.

The DTPAA is added to the plasmid digest and stirred at room temperature for at least 60 minutes. After storage overnight at 4 °C a lanthanide salt (*e.g.*, terbium chloride) is added to the reaction mixture. The resulting mixture is shaken and allowed to stand for at least 30 minutes. Excess hydrolyzed chelate and lanthanide salt can be separated from the plasmid digest-chelate conjugate by passing the mixture through a column packed with Sephadex, such as Sephadex G 25-150. Suitable elution buffers include, for example, 10 mM 3-[N-morpholino]propane sulfonic acid at pH 7. The DNA fractions can then be pooled and evaporated to dryness. The DNA fractions can then be loaded into a microfluidic device and sequenced.

The plasmid digest-chelate conjugate can be characterized by determining the DNA concentration by measuring the absorbance at 260 nm. Label concentration can be determined by comparing the fluorescence of the purified labeled nucleotide conjugate with the fluorescence of the free chelate complexed with terbium. Suitable

instrumentation for these measurements includes a Perkin-Elmer Lambda Array UV-Vis spectrometer and a Perkin-Elmer LS-5 spectrofluorimeter.

Other exemplary methods of attaching fluorescent labels onto nucleic acids are taught in U.S. Patent No. 5,721,355 and Chehab and Kan, *Proc. Nat'l. Acad. Sci. USA* **86**:9178-9182 (1989).

E. MICROFLUIDIC DEVICES

A variety of microscale systems and/or components which can be adapted to the present invention by incorporating assay components and appropriate additional elements, e.g., related to signal detection as noted herein, are available. Microfluidic devices, which can be adapted to the present invention by the addition of signal detection elements or addition of fluorescent components as set forth herein, are described in various PCT applications and issued U.S. Patents by the inventors and their coworkers, including U.S. Patent Nos. 5,699,157 (J. Wallace Parce) issued 12/16/97; 5,779,868 (J. Wallace Parce et al.) issued 07/14/98; 5,800,690 (Calvin Y.H. Chow et al.) issued 09/01/98; and 5,842,787 (Anne R. Kopf-Sill et al.) issued 12/01/98; and published PCT applications, such as, WO 98/00231, WO 98/00705, WO 98/00707, WO 98/02728, WO 98/05424, WO 98/22811, WO 98/45481, WO 98/45929, WO 98/46438, and WO 98/49548.

For example, pioneering technology providing high-throughput microscale assays are set forth in Parce et al. "HIGH THROUGHPUT SCREENING ASSAY SYSTEMS IN MICROSCALE FLUIDIC DEVICES" WO 98/00231 and, e.g., in 60/128,643 filed April 4, 1999, entitled "MANIPULATION OF MICROPARTICLES IN MICROFLUIDIC SYSTEMS," by Mehta et al. Complete integrated systems with fluid handling, signal detection, sample storage and sample accessing are available. For example, Parce et al. "HIGH THROUGHPUT SCREENING ASSAY SYSTEMS IN MICROSCALE FLUIDIC DEVICES" WO 98/00231 provide pioneering technology for the integration of microfluidics and sample selection and manipulation.

Typically, the microfluidic systems of the invention provide an integration of several elements, including a microfluidic device with interior microfluidic channels and reservoirs, optics for viewing labeled components, computer systems and software for recording and analyzing components and the like. WO 98/00231 entitled "HIGH THROUGHPUT SCREENING ASSAY SYSTEMS IN MICROSCALE FLUIDIC DEVICES" by Parce *et al.* provides pioneering technology related to microscale fluidic

devices, including electrokinetic devices. The devices are generally suitable for assays relating to the interaction of biological and chemical species, including enzymes and substrates, ligands and ligand binders, receptors and ligands, antibodies and antibody ligands, as well as many other assays. Because the devices provide the ability to mix fluidic reagents and assay mixing results in a single continuous process, and because minute amounts of reagents can be assayed, these microscale devices represent a fundamental advance for laboratory science. Pioneering integrated systems for nucleic acid sequencing and other iterative fluid manipulation processes utilizing microfluidic fluid manipulation are described in, *e.g.*, in "CLOSED LOOP BIOCHEMICAL ANALYZERS" by Knapp *et al.*, WO98/45481.

In the integrated systems provided by Knapp, pioneering iterative methods, including nucleic acid sequencing methods as applied to microfluidic systems are described in detail. These systems are applicable to the present invention, *i.e.*, by using the systems to produce sequencing reaction products and then measuring fluorescent decay times of sequencing products as described *supra*. In these embodiments, standard primer walking sequencing strategies are automated and integrated. All of the mixing and analysis steps for sequencing are optionally performed with an integrated system.

In brief, a template nucleic acid is selected and introduced into a reaction channel in a microfluidic device of the invention. The template is optionally amplified, *e.g.*, by introducing PCR or LCR reagents into the channel and performing cycles of heating and cooling on the template. Thermocycling in microscale devices is described in USSN 60/083,532, attorney docket number 100/01320 entitled "ELECTRICAL CURRENT FOR CONTROLLING FLUID TEMPERATURES IN MICROCHANNELS" filed April 29, 1998 by Calvin Chow, Anne R. Kopf-Sill and J. Wallace Parce and in related application 08/977,528, filed November 25, 1997. A variety of "joule heating" schemes for PCR in microfluidic systems are taught in WO 98/17910 and in WO 98/45481. Examples of non-thermocyclic polymerase mediated reactions are also taught in WO 98/45481. Methods and microfluidic apparatus for performing microscale PCR, including thermocycling by approaches other than joule heating, are also taught in 09/287,069 (INEFFICIENT FAST PCR) by Kopf-Sill.

As previously noted, in joule heating approaches, energy is provided to heat fluids, *e.g.*, samples, analytes, sequencing buffers and reagents, in desired locations

of the substrates in an efficient manner by application of electric current to fluids in microchannels. Thus, the present invention optionally uses power sources that pass electrical current through the fluid in a channel for heating purposes, as well as for material transport (in alternate embodiments, heating is produced by application of external heating or cooling sources, or the like). In exemplary embodiments, the fluid passes through a channel of a desired cross-section (*e.g.*, diameter) to enhance thermal transfer of energy from the current to the fluid. The channels can be formed on almost any type of substrate material such as, for example, amorphous materials (*e.g.*, glass, plastic, silicon), composites, multi-layered materials, combinations thereof, and the like.

Alternatively, *e.g.*, where the source of template is from an abundant sequence such as a cloned nucleic acid, further amplification can be unnecessary. However, in addition to amplification procedures, there are a number of PCR-based sequencing methods available, such as a PCR nuclease chain termination procedure that can also be used for direct sequencing in the methods of the invention, by incorporating fluorescent nucleotides which are distinguishable by their decay time into templates for sequencing. Porter *et al.* (1997) *Nucleic Acids Research* 25(8):1611-1617 describe the biochemistry of exemplar PCR chain termination methods.

Sequencing reagents are added to the template nucleic acid, e.g., by electrokinetic or pressure-based flow of reagents into contact with the template nucleic acid, e.g., in a reaction channel, and a sequencing reaction is performed appropriate to the particular reaction in use. Many appropriate reactions are known, with the Sanger dideoxy chain termination method being the most common. In integrated systems, the primer used to prime synthesis is optionally selected from a pre-synthesized set of nucleic acid primers, preferably a set including many or all of the primers for a particular primer length. In one aspect, modular primers are used. *See,* Beskin *et al.* (1995) *Nucleic Acids Research* 23(15):2881-2885 and Raja *et al.* (1997) *Nucleic Acids Research* 25(4):800-805 for a description of modular primers. *See,* Knapp *et al.*, *supra* for a description of the use of modular primers in microfluidic systems. According to the present invention, these primers can incorporate fluorescent labels distinguishable by their decay times, or nucleotides incorporated by primer extension can incorporate such labels.

After the sequencing or other reaction is run, products are separated by size and/or charge in an analysis region of the microfluidic device. Devices of the invention can be used to electrophoretically separate macromolecules by size and/or

charge. The separated products are detected as they pass a fluorescent detector (nucleic acids and other molecules are typically labeled with fluorophores that are distinguishable by their decay times in the present invention; accordingly, appropriate detectors include spectrophotometers, fluorescent detectors, microscopes (*e.g.*, for fluorescent microscopy), etc. As applied to the present invention, the detection systems are adapted to measure fluorescence decay times.

In sequencing reactions, detection of the size separated products is used to compile sequence information for the region being sequenced. A computer is optionally used to select a second primer from the pre-synthesized primer set which hybridizes to the sequenced region, and the process is iteratively repeated with the second primer, leading to sequencing of a second region, selection of a third primer hybridizing to the second region, *etc*. A variety of commercially available hardware and software is available for digitizing, storing, and analyzing a signal or image such as that generated by the microfluidic device described herein. Typically, a computer commonly used to transform signals from the detection device into reaction rates will be a PC™-compatible computer (*e.g.*, having a central processing unit (CPU) compatible with x86 CPUs, and running an operating system such as DOS™, OS/2 Warp™, WINDOWS/NT™, or WINDOWS 95™), a Macintosh™ (running MacOS™), or a UNIX workstation (*e.g.*, a SUN™ workstation running a version of the Solaris™ operating system, or PowerPC™ workstation) are all commercially common, and known to one of skill in the art. Data analysis software on the computer is then employed to determine the rate of formation and or mobility of any component which is labeled with a fluorescent label distinguishable by its decay time. Software for determining reaction rates is available, or can easily be constructed by one of skill using a standard programming language such as Visual Basic, Fortran, Basic, Java, C, Visual C++or the like. It will be recognized that the specific configuration of integrated devices and systems will generally vary depending upon the type of manipulation or reaction to be performed. The small scale, integratability and self-contained nature of the microfluidic elements of these devices and systems allows for virtually any reaction or separation to be performed.

Finally, it should be appreciated that the use and detection of fluorescent labels which are distinguishable by their decay times can be applied in microfluidic systems to a variety of problems other than sequencing. Essentially any fluorogenic or

non-fluorogenic assay can be practiced using fluorescent labels which are distinguishable by their decay times. For example, Kopf-Sill *et al.* WO98/56956 "APPARATUS AND METHODS FOR CORRECTING FOR VARIABLE VELOCITY IN MICROFLUIDIC SYSTEMS" provides a variety of fluorogenic and non-fluorogenic assay formats for microfluidic systems which can be adapted to the present invention by the incorporation of decay-time distinguishable labels. *See also,* A. R. Kopf-Sill, T. Nikiforov, L. Bousse, R. Nagel, & J. W. Parce, "Complexity and performance of on-chip biochemical assays," in Proceedings of Micro- and Nanofabricated Electro-Optical Mechanical Systems for Biomedical and Environmental Applications, SPIE, Vol. 2978, San Jose, California, February 1997, p. 172-179). These include a variety of reactants and products assessed in microfluidic systems by detection of fluorescent or non-fluorescent labels, including ligands and ligand binders such as an antibody and an antibody ligand, receptors and receptor ligands, biotin and avidin, proteins and complementary binding proteins, carbohydrates and carbohydrate binding moieties, nucleic acids, *etc.*

Reactants or, e.g., molecules which hybridize, are contacted by flowing the components together in a microfluidic system. At least one of the components is typically labeled with a label distinguishable by its decay time. Products and reactants are detected and quantitated by observing, *e.g.*, the movement of labels in the system. Data correction for the effects of velocity of components can be applied, *e.g.*, by considering conservation of flux in the flowing systems, by generating and applying data masking files, by using self-correcting fluid sampling techniques and the like. *See*, Kopf-Sill *et al., supra.*

FLUID MOVEMENT IN MICROSCALE SYSTEMS

A variety of fluid movement approaches can be used in microscale systems. For example, pressure forces can be applied to microscale elements to achieve fluid movement using any of a variety of techniques. Fluid flow (and flow of materials suspended or solubilized within the fluid, including sequencing reagents, enzymes, enzyme substrates, catalysts, cells or other particles, etc.) is optionally regulated by pressure based mechanisms such as those based upon fluid displacement, e.g., using a piston, pressure diaphragm, vacuum pump, probe or the like to displace liquid and raise or lower the pressure at a site in the microfluidic system. The pressure is optionally pneumatic, e.g., a pressurized gas, or uses hydraulic forces, e.g., pressurized liquid, or alternatively, uses a positive or negative displacement mechanism, i.e., a plunger fitted

into a material reservoir, for forcing material through a channel or other conduit, or is a combination of such forces.

In one class of preferred embodiments, a vacuum source is applied to a reservoir or well at one end of a channel to draw the relevant materials through the channel (e.g., fluidic compositions comprising enzymes, buffers, substrates, reaction modulators, sequencing reagents or the like). Pressure or vacuum sources are optionally supplied external to the device or system, e.g., external vacuum or pressure pumps sealably fitted to the inlet or outlet of the channel, or they are internal to the device, e.g., microfabricated pumps integrated into the device and operably linked to the channel. Examples of microfabricated pumps have been widely described in the art. *See, e.g.*, published International Application No. WO 97/02357.

Hydrostatic, wicking and capillary forces can also be used to provide pressure for fluid flow of materials such as cells, biological molecules, particles and chemicals. See, e.g., "METHOD AND APPARATUS FOR CONTINUOUS LIQUID FLOW IN MICROSCALE CHANNELS USING PRESSURE INJECTION, WICKING AND ELECTROKINETIC INJECTION," by Alajoki et al. USSN 09/245,627, filed February 5, 1999. In these methods, an adsorbent material or branched capillary structure is placed in fluidic contact with a region where pressure is applied, thereby causing fluid to move towards the adsorbent material or branched capillary structure.

In an alternate embodiment, microfluidic systems can be incorporated into centrifuge rotor devices, which are spun in a centrifuge. Fluids and particles travel through the device due to gravitational and centripetal/centrifugal pressure forces.

One preferred method of achieving transport or movement of modulators and modulator target components through microfluidic channels is by electrokinetic material transport. "Electrokinetic material transport systems," as used herein, include systems that transport and direct materials within a microchannel and/or chamber containing structure, through the application of electrical fields to the materials, thereby causing material movement through and among the channel and/or chambers, i.e., cations will move toward a negative electrode, while anions will move toward a positive electrode. For example, movement of fluids toward or away from a cathode or anode can cause movement of sequencing reagents or products or other biological or relevant molecules suspended within the fluid. Similarly, the reagents, etc. can be charged, in which case they will move toward an oppositely charged electrode (indeed, in this case, it

is possible to achieve fluid flow in one direction while achieving particle flow in the opposite direction). In this embodiment, the fluid can be immobile or flowing and can comprise a matrix as in electrophoresis.

In general, electrokinetic material transport and direction systems also include those systems that rely upon the electrophoretic mobility of charged species within the electric field applied to the structure. Such systems are more particularly referred to as electrophoretic material transport systems. For electrophoretic applications, the walls of interior channels of the electrokinetic transport system are optionally charged or uncharged. Typical electrokinetic transport systems are made of glass, charged polymers, and uncharged polymers. The interior channels are optionally coated with a material which alters the surface charge of the channel.

Use of electrokinetic transport to control material movement in interconnected channel structures was described, e.g., in WO 96/04547 and US 5,858,195 to Ramsey. An exemplary controller is described in U.S. 5,800,690. Modulating voltages are concomitantly applied to the various reservoirs to affect a desired fluid flow characteristic, e.g., continuous or discontinuous (e.g., a regularly pulsed field causing the sample to oscillate a direction of travel) flow of labeled components toward a waste reservoir. Particularly, modulation of the voltages applied at the various reservoirs can move and direct fluid flow through the interconnected channel structure of the device.

As discussed above, any of a number of methods and devices are suitable for use in the present invention for separating the components of a mixture; however, typically, the methods of the invention are practiced in the context of a microfluidic system. Fluorescently labeled components (typically multiple components comprising one or more labels distinguishable by their decay time) are transported through a microfluidic channel. Material transport and direction in the microfluidic channel is typically accomplished through electrokinesis, *e.g.*, electroosmosis or electrophoresis or by pressure-based fluid movement, or a combination thereof.

Thus, in a preferred embodiment, molecular separation is performed using a microfluidic apparatus. A preferred microfluidic apparatus has a body structure with at least two intersecting channels fabricated inside the body structure, as taught in the numerous references regarding microfluidic technology noted above. The channels

preferably have at least one cross-sectional dimension that is in the range of from about 0.1 to about 500 μm.

In other preferred embodiments, when a sequencing reaction is used, in certain preferred embodiments, the sequencing reaction is performed using the microfluidic device. The microfluidic device also comprises a detecting station and the device is used for both separating and detecting the components of a mixture.

In yet a further preferred embodiment, the microfluidic device is also used for identifying the compounds of the mixture by the differences in their fluorescence lifetimes. In this embodiment, the invention provides a microfluidic device with at least one microchannel, a detector for detecting fluorescence species in the channel and a digital computer which is operatively linked to the detector. The digital computer is used to determine the lifetimes of the fluorescent species.

Preferred arrangements for fluid movement and monitoring of fluorescent decay times are described above, e.g., in the figures.

Controllers

A variety of controlling instrumentation is optionally utilized in conjunction with the microfluidic devices described above, for controlling the transport and direction of fluids and/or materials within the devices of the present invention, e.g., by pressure-based and/or electrokinetic control.

For example, in many cases, fluid transport and direction are controlled in whole or in part, using pressure based flow systems that incorporate external or internal pressure sources to drive fluid flow. Internal sources include microfabricated pumps, e.g., diaphragm pumps, thermal pumps, lamb wave pumps and the like that have been described in the art. See, e.g., U.S. Patent Nos. 5,271,724, 5,277,556, and 5,375,979 and Published PCT Application Nos. WO 94/05414 and WO 97/02357. As noted above, the systems described herein can also utilize electrokinetic material direction and transport systems.

Preferably, in systems utilizing pressure-based control, external pressure sources are used, and applied to ports at channel termini. These applied pressures, or vacuums, generate pressure differentials across the lengths of channels to drive fluid flow through them. In the interconnected channel networks described herein, differential flow rates on volumes are optionally accomplished by applying different pressures or vacuums

at multiple ports, or preferably, by applying a single vacuum at a common waste port and configuring the various channels with appropriate resistance to yield desired flow rates. Example systems are described in USSN 09/238,467, filed 1/28/99.

In addition, the use of electrokinetic transport to control material movement in interconnected channel structures is an alternate preferred method of achieving material transport (and controllers can include both pressure-based and electrokinetic control elements). This method of material control is particularly useful in electrophonetic separation of labeled components. For example, electrokinetic material transport is set forth, e.g., in WO 96/04547 and US 5,858,195 to Ramsey, as well as in a variety of other references noted herein. An exemplary electrokinetic controller is described in U.S. 5,800,690. During electrokinetic control, modulating voltages are concomitantly applied to the various reservoirs to affect a desired fluid or material flow characteristic, e.g., continuous or discontinuous (e.g., a regularly pulsed field causing the sample to oscillate direction of travel) flow of labeled components toward a waste reservoir. Particularly, modulation of the voltages applied at the various reservoirs can move and direct fluid flow through the interconnected channel structure of the device.

Typically, controller systems are appropriately configured to receive or interface with a microfluidic device or system element as described herein. For example, the controller and/or detector, optionally includes a stage upon which the device of the invention is mounted to facilitate appropriate interfacing between the controller and/or detector and the device. Typically, the stage includes an appropriate mounting/alignment structural element, such as a nesting well, alignment pins and/or holes, asymmetric edge structures (to facilitate proper device alignment), and the like. Many such configurations are described in the references cited herein.

The controlling instrumentation discussed above is also used to provide for pressure-based or electrokinetic injection or withdrawal of material downstream of the region of interest to control an upstream flow rate. The same instrumentation and techniques described above are also utilized to inject a fluid into a downstream port to function as a flow control element.

ADDITIONAL FEATURES

In additional aspects, the present invention provides for the use of any of the apparatus elements described herein, e.g., for practicing any of the methods or assays set forth herein.

The invention provides for the use of a fluorescent molecule in any of the assays set forth herein, and for the use of the apparatus herein to detect such molecules.

Kits are provided which incorporate, e.g., any of the apparatus elements herein, e.g., in conjunction with instructions for practicing the methods herein, packaging, reagents, fluorescent molecules such as control molecules, containers for holding apparatus or reagent elements, or the like.

While the invention has been described by way of example and in terms of the specific embodiments, it is to be understood that the invention is not limited to the disclosed embodiments. To the contrary, the scope of the invention includes, e.g., various modifications and similar arrangements as would be apparent to those skilled in the art. Therefore, the scope of the appended claims should be accorded the broadest interpretation so as to encompass all such modifications and similar arrangements, as should any claims added during prosecution. The disclosures of all articles and references, including patent applications and publications, cited herein are incorporated herein by reference for all purposes as though each reference were separately indicated to be incorporated by reference for all purposes.

WHAT IS CLAIMED IS:

1. An apparatus for use in measuring the fluorescent lifetimes of a plurality of fluorescent labels, wherein the labels are provided to a detection region of the apparatus, the apparatus comprising:

a radiation source, the radiation source emitting radiation for irradiating the detection region, wherein the radiation excites the fluorescent labels and causes the labels to fluoresce;

a fluorescence detector for detecting the fluorescent emissions from the fluorescent labels in the detection region, wherein each one of the plurality of fluorescent labels has a different fluorescent lifetime; and

a processor, coupled to the fluorescence detector, for analyzing the fluorescent emissions to determine the fluorescent lifetimes of the labels.

2. The apparatus of claim 1, further comprising a beamsplitter positioned between the detection region and the radiation source such that a substantial portion of the radiation emitted from the radiation source passes to the detection region, and a substantial portion of radiation incident from the detection region is redirected toward the fluorescence detector.

3. The apparatus of claim 1, further comprising a beamsplitter positioned between the detection region and the radiation source, wherein the beamsplitter allows a substantial portion of the radiation emitted from the radiation source having a wavelength below a threshold wavelength to pass, and reflects toward the fluorescence detector a substantial portion of radiation incident from the detection region having a wavelength above the threshold wavelength.

4. The apparatus of claim 3, wherein the threshold wavelength is approximately 650nm.

5. The apparatus of claim 1, further comprising a sample in the detection region of the apparatus including at least one of the plurality of fluorescent labels.

6. The apparatus of claim 1, further comprising a microfluidic substrate holder positioned to mount a microfluidic substrate proximal to the detection region.

7. The apparatus of claim 6, further comprising a microfluidic substrate mounted in the microfluidic substrate holder, the microfluidic substrate including at least two intersecting microchannels, wherein at least a portion of at least one of the at least two microchannels is exposed to the radiation emitted from the radiation source.

8. The apparatus of claim 7, further comprising electrokinetic control means for controlling the flow of material through the microchannels.

9. The apparatus of claim 1, further comprising a modulator coupled to the radiation source, wherein the radiation source emits modulated radiation in response to a reference signal generated by the modulator.

10. The apparatus of claim 9, wherein the modulator includes an oscillator, wherein the processor includes a phase shift detector coupled to the oscillator, wherein each fluorescent emission is modulated, wherein the fluorescence detector generates first signals proportional to the fluorescent emissions, and wherein the phase shift detector compares the first signals with the reference signal generated by the oscillator to determine at least one of the phase shift between the first signals and the reference signal and the amount of modulation of the first signals.

11. The apparatus of claim 1, further comprising a chopper positioned between the detection region and the radiation source, wherein the chopper operates to chop the emitted radiation such that the emitted radiation irradiates the detection region in the form of pulses.

12. The apparatus of claim 1, further comprising a pulse generator coupled to the radiation source, wherein the radiation source emits radiation pulses in response to a reference signal generated by the pulse generator.

13. The apparatus of claim 1, wherein the processor includes a pulse decay time detector, wherein the fluorescent emissions are in the form of decaying pulses,

and wherein the pulse decay time detector measures the decay times of the fluorescent emissions.

14. The apparatus of claim 1, further comprising optical elements, positioned between the detection region and the radiation source, for collimating and focusing the emitted radiation onto the detection region.

15. The apparatus of claim 1, wherein the radiation source is selected from the group consisting of a laser, a laser diode, a light emitting diode (LED), an arc lamp and a flashlamp.

16. The apparatus of claim 1, wherein the fluorescence detector is selected from the group consisting of an avalanche photo diode, a photo multiplier tube, and a photo diode.

17. An apparatus for use in determining the sequence of nucleotides in a nucleic acid sample having a plurality of overlapping nucleic acid fragments, wherein each fragment includes one of four different fluorescent labels, each label having a different fluorescent lifetime, each of the labels binding to a specific nucleotide, wherein the nucleic acid fragments of the nucleic acid sample are provided to a detection region, the apparatus comprising:

a radiation source, the radiation source emitting radiation for irradiating the detection region, wherein the radiation excites the fluorescent labels and causes the labels to fluoresce;

a fluorescence detector for detecting the fluorescent emissions from the fluorescent labels in the detection region; and

a processor, coupled to the fluorescence detector, for analyzing the fluorescent emission to determine the fluorescent lifetimes of the labels, thereby determining the sequence of nucleotides in the nucleic acid.

18. The apparatus of claim 17, further comprising a beamsplitter positioned between the detection region and the radiation source such that a substantial portion of the radiation emitted from the radiation source passes to the detection region, and a substantial portion of radiation incident from the detection region is redirected toward the fluorescence detector.

19. The apparatus of claim 17, further comprising a beamsplitter positioned between the detection region and the radiation source, wherein the beamsplitter allows a substantial portion of the radiation emitted from the radiation source having a wavelength below a threshold wavelength to pass, and reflects toward the fluorescence detector a substantial portion of radiation incident from the detection region having a wavelength above the threshold wavelength.

20. The apparatus of claim 19, wherein the threshold wavelength is approximately 650nm.

21. The apparatus of claim 17, further comprising a microfluidic substrate holder positioned to mount a microfluidic substrate proximal to the detection region.

22. The apparatus of claim 21, further comprising a microfluidic substrate mounted in the microfluidic substrate holder, the microfluidic substrate including at least two intersecting microchannels, wherein at least a region of at least one of the at least two microchannels is exposed to the radiation emitted from the radiation source.

23. The apparatus of claim 22, further comprising electrokinetic control means for controlling the flow of material through the microfluidic channels.

24. The apparatus of claim 17, wherein the fluorescence detector is selected from the group consisting of an avalanche photo diode, a photo multiplier tube, and a photo diode.

25. The apparatus of claim 17, further comprising a modulator coupled to the radiation source, wherein the radiation source emits modulated radiation in response to a reference signal generated by the modulator.

26. The apparatus of claim 25, wherein the modulator includes an oscillator, wherein the processor includes a phase shift detector coupled to the oscillator, wherein each fluorescent emission is modulated, wherein the fluorescence detector generates first signals proportional to the fluorescent emission, and wherein the phase

shift detector compares the first signals with the reference signal generated by the oscillator to determine at least one of the phase shift between the first signals and the reference signal and the amount of modulation of the first signals.

27. The apparatus of claim 17, further comprising a chopper positioned between the detection region and the radiation source, wherein the chopper operates to chop the emitted radiation such that the emitted radiation irradiates the detection region in the form of pulses.

28. The apparatus of claim 17, further comprising a pulse generator coupled to the radiation source, wherein the radiation source emits radiation pulses in response to a reference signal generated by the pulse generator.

29. The apparatus of claim 17, wherein the processor includes a pulse decay time detector, wherein the fluorescent emissions are in the form of decaying pulses, and wherein the pulse decay time detector measures the decay times of the fluorescent emissions.

30. The apparatus of claim 17, further comprising optical elements, positioned between the detection region and the radiation source, for collimating and focusing the emitted radiation onto the detection region.

31. The apparatus of claim 17, wherein the radiation source is selected from the group consisting of a laser, a laser diode, a light emitting diode (LED), an arc lamp and a flashlamp.

32. A system for measuring the fluorescent lifetimes of a plurality of fluorescent labels, the system comprising:

a detection region, wherein the labels are provided to the detection region;

a radiation source, the radiation source emitting radiation for irradiating the detection region, wherein the radiation excites the fluorescent labels and causes the labels to fluoresce;

detection means for detecting the fluorescent emissions from the fluorescent labels in the detection region; and

means, coupled to the fluorescence detector, for analyzing the fluorescent emissions to determine the fluorescent lifetimes of the labels.

33. The system of claim 32, further comprising a beamsplitter positioned between the detection region and the radiation source such that a substantial portion of the radiation emitted from the radiation source passes to the detection region, and a substantial portion of radiation incident from the detection region is redirected toward the detection means.

34. The system of claim 32, further comprising a beamsplitter positioned between the detection region and the radiation source, wherein the beamsplitter allows a substantial portion of the radiation emitted from the radiation source having a wavelength below a threshold wavelength to pass, and causes a substantial portion of radiation incident from the detection region having a wavelength above the threshold wavelength to reflect toward the detection means.

35. The system of claim 34, wherein the threshold wavelength is approximately 650nm.

36. The system of claim 32, further comprising a sample in the detection region, the sample including at least one of the plurality of fluorescent labels.

37. The system of claim 32, further comprising a microfluidic substrate holder positioned to mount a microfluidic substrate proximal to the detection region.

38. The system of claim 37, further comprising a microfluidic substrate mounted in the microfluidic substrate holder, the microfluidic substrate including at least two intersecting microchannels, wherein at least a portion of at least one of the at least two microchannels is exposed to the radiation emitted from the radiation source.

39. The system of claim 38, further comprising electrokinetic control means for controlling the flow of material through the microchannels.

40. The system of claim 32, wherein the radiation source is selected from the group consisting of a laser, a laser diode, a light emitting diode (LED), an arc lamp and a flashlamp.

41. The system of claim 32, wherein the detection means includes a detector selected from the group consisting of an avalanche photo diode, a photo multiplier tube, and a photo diode.

42. The system of claim 32, further comprising modulation means coupled to the radiation source for modulating the radiation source such that the radiation source emits modulated radiation.

43. The system of claim 42, wherein the modulation means includes an oscillator, wherein the analyzing means includes a phase shift detector coupled to the modulation means, wherein each fluorescent emission is modulated, wherein the detection means generates first signals proportional to the fluorescent emission, and wherein the phase shift detector compares the first signals with a reference signal generated by the oscillator to determine at least one of the phase shift between the first signals and the reference signal and the amount of modulation of the first signals.

44. The system of claim 43, wherein the phase shift detector includes a phase lock loop (PLL) for measuring the phase differences between the first signals and the reference signal.

45. The system of claim 32, further comprising pulse generation means for causing the radiation emitted from the radiation source to irradiate the detection region in the form of pulses.

46. The system of claim 45, wherein the analyzing means includes a pulse decay time detector, wherein the fluorescent emissions are in the form of decaying pulses, and wherein the pulse decay time detector measures the decay times of the fluorescent emissions.

47. The system of claim 46, wherein the pulse decay time detector is coupled to the pulse generation means, wherein the detection means generates first signals proportional to the decaying fluorescent emissions, and wherein the pulse generation means provides a reference signal to the pulse decay time detector for comparison with the first signals.

48. The system of claim 45, wherein the pulse generation means includes a chopper positioned between the detection region and the radiation source.

49. The system of claim 45, wherein the pulse generation means includes a pulse generator coupled to the radiation source, wherein the radiation source emits radiation pulses in response to a reference signal generated by the pulse generator.

50. The system of claim 32, further comprising means, positioned between the detection region and the radiation source, for collimating and focusing the emitted radiation onto the detection region.

51. The system of claim 32, further including a nucleic acid sample having a plurality of nucleic acid fragments, wherein each nucleic acid fragment includes one of four different fluorescent labels, each label having a different fluorescent lifetime, each of the fluorescent labels binding to a specific nucleotide in the nucleic acid sample, wherein the nucleic acid sample is provided to the detection region, and wherein the analyzing means analyzes the fluorescent emission to determine the fluorescent lifetimes of the labels, thereby determining the sequence of nucleotides in the nucleic acid.

52. The system of claim 51, wherein the nucleic acid fragments are provided to the detection region in an order, and wherein the analyzing means analyzes the fluorescent emissions in said order.

53. A method of measuring the fluorescent lifetimes of a plurality of fluorescent labels, the method comprising the steps of:

providing the labels to a detection region;

irradiating the detection region with radiation emitted from a radiation source, wherein the radiation excites the fluorescent labels and causes the labels to fluoresce;

detecting the fluorescent emissions of the fluorescent labels using a fluorescence detector, wherein each one of the plurality of fluorescent labels has a different fluorescent lifetime; and

analyzing the fluorescent emissions using a processor coupled to the fluorescence detector to determine the fluorescent lifetimes of the labels.

54. The method of claim 53, further comprising the step of positioning a beamsplitter between the detection region and the radiation source such that a substantial portion of the radiation emitted from the radiation source passes to the detection region, and a substantial portion of radiation incident from the detection region is redirected toward the fluorescence detector.

55. The method of claim 53, further comprising the step of positioning a beamsplitter between the detection region and the radiation source, wherein the beamsplitter allows a substantial portion of the radiation emitted from the radiation source having a wavelength below a threshold wavelength to pass, and causes a substantial portion of radiation incident from the detection region having a wavelength above the threshold wavelength to reflect toward the fluorescence detector.

56. The method of claim 55, wherein the threshold wavelength is approximately 650nm.

57. The method of claim 53, further comprising mounting a microfluidic substrate in a microfluidic substrate holder, the microfluidic substrate including at least two intersecting microchannels, wherein at least a portion of at least one of the at least two microchannels is exposed to the radiation emitted from the radiation source, wherein the step of providing includes providing the labels to the exposed portion.

58. The method of claim 57, wherein the step of providing includes applying voltages to the microchannels so as to control the movement of material through the microchannels.

59. The method of claim 53, wherein the radiation source is selected from the group consisting of a laser, a laser diode, a light emitting diode (LED), an arc lamp and a flashlamp.

60. The method of claim 53, wherein the fluorescence detector is selected from the group consisting of an avalanche photo diode, a photo multiplier tube, and a photo diode.

61. The method of claim 53, further comprising the step of modulating the radiation source with a reference signal such that the radiation source emits modulated radiation.

62. The method of claim 61, wherein the fluorescence detector generates first signals proportional to the detected fluorescent emissions, wherein each fluorescent emission is modulated, and wherein the step of analyzing includes:

using a phase shift detector to compare the first signals with the reference signal to determine at least one of the phase shift between the first signals and the reference signal and the amount of modulation of the first signals.

63. The method of claim 62, wherein the phase shift detector includes a phase lock loop (PLL) for measuring the phase differences between the first signals and the reference signal.

64. The method of claim 53, further comprising the step of modulating the radiation source with a pulse generator such that the radiation source emits radiation pulses.

65. The method of claim 64, wherein the fluorescence detector generates first signals proportional to the detected fluorescent emissions, wherein the fluorescent emissions are in the form of decaying pulses, and wherein the step of analyzing includes:

using a detector to measure the decay times of the fluorescent emissions.

66. The method of claim 65, wherein the detector is a pulse decay time detector which is coupled to the pulse generator, and wherein the pulse generator provides a reference signal to the pulse decay time detector for comparison with the decaying fluorescent pulses.

67. The method of claim 53, further comprising collimating and focusing the emitted radiation onto the detection region.

68. The method of claim 53, further comprising chopping the emitted radiation with a chopper such that the emitted radiation irradiates the detection region in the form of pulses.

69. The method of claim 68, wherein the fluorescence detector generates first signals proportional to the detected fluorescent emissions, wherein the fluorescent emissions are in the form of decaying pulses, and wherein the step of analyzing includes:

using a detector to measure the decay times of the fluorescent emissions.

70. The method of claim 53, wherein the step of providing the labels to the detection region includes providing a nucleic acid sample to the detection region, the nucleic acid sample having a plurality of nucleic acid fragments, wherein each nucleic acid fragment includes one of four different fluorescent labels, each label having a different fluorescent lifetime, each of the four different fluorescent labels binding to a specific nucleotide.

71. The method of claim 70, wherein the nucleic acid fragments of the nucleic acid sample are provided to the detection region in an order.

72. The method of claim 70, wherein the step of analyzing the fluorescent emissions includes determining the sequence of nucleotides in the nucleic acid sample based on the fluorescent lifetimes of the labels.

73. An apparatus for use in measuring the fluorescent lifetimes of a plurality of fluorescent labels, wherein the labels are provided to a microfluidic detection region of the apparatus, the apparatus comprising:

a radiation source, the radiation source emitting radiation for irradiating the microfluidic detection region, wherein the radiation excites the fluorescent labels and causes the labels to fluoresce;

a fluorescence detector for detecting the fluorescent emissions from the fluorescent labels in the microfluidic detection region, wherein each one of the plurality of fluorescent labels has a different fluorescent lifetime; and

a processor, coupled to the fluorescence detector, for analyzing the fluorescent emissions to determine the fluorescent lifetimes of the labels.

74. A method of distinguishing between a plurality of fluorescent species, wherein each of said fluorescent species has a fluorescence emission, said emission having a characteristic fluorescence lifetime, said method comprising:

(a) electrokinetically transporting each of said species through a microfluidic channel;

(b) detecting each of said fluorescent species in said channel; and

(c) identifying each of said fluorescent species by measuring said characteristic fluorescence lifetime.

75. The method according to claim 74, wherein said microfluidic channel is in a microfluidic apparatus comprising two or more intersecting channels.

76. The method according to claim 75, wherein said microfluidic apparatus comprises:

a glass or polymer body having at least two intersecting channels fabricated therein, at least one of said at least two intersecting channels having at least one cross-sectional dimension in the range of from about 0.1 μm to about 500 μm.

77. A method of sequencing a nucleic acid polymer of interest, said method comprising:

(a) performing a sequencing reaction on said nucleic acid polymer utilizing a sequencing reaction mixture comprising said nucleic acid polymer and a first labeled nucleic acid bearing a first fluorescent label, wherein said fluorescent label has a fluorescence emission, said emission having a characteristic fluorescence lifetime;

(b) electrokinetically transporting each of said species through a microfluidic channel;

(c) detecting said first labeled nucleic acid bearing a first fluorescent label; and

(d) identifying said first labeled nucleic acid bearing a first fluorescent label by measuring said characteristic fluorescence lifetime.

78. The method according to claim 77, wherein said sequencing reaction mixture further comprises a second labeled nucleic acid bearing a second fluorescent label, wherein said second fluorescent label has a fluorescence emission, said emission having a characteristic fluorescence lifetime.

79. The method according to claim 78, wherein said sequencing reaction mixture further comprises a third labeled nucleic acid bearing a third fluorescent label,

wherein said third fluorescent label has a fluorescence emission, said emission having a characteristic fluorescence lifetime.

80. The method according to claim 79, wherein said sequencing reaction mixture further comprises a fourth labeled nucleic acid bearing a fourth fluorescent label, wherein said fourth fluorescent label has a fluorescence emission, said emission having a characteristic fluorescence lifetime.

81. The method according to claim 77, wherein said first labeled nucleic acid bearing a first fluorescent label is a member of a plurality of unique labeled nucleic acid species, wherein each unique labeled nucleic acid species comprises a unique fluorescent label having a fluorescence emission, said emission having a characteristic fluorescence lifetime.

82. The method according to claim 77 further comprising a second sequencing reaction mixture comprising said nucleic acid polymer of interest and a second labeled nucleic acid bearing a second fluorescent label, wherein said second fluorescent label has a fluorescence emission, said emission having a characteristic fluorescence lifetime.

83. The method according to claim 82 further comprising a third sequencing reaction mixture comprising said nucleic acid polymer of interest and a third labeled nucleic acid bearing a third fluorescent label, wherein said third fluorescent label has a fluorescence emission, said emission having a characteristic fluorescence lifetime.

84. The method according to claim 83 further comprising a fourth sequencing reaction mixture comprising said nucleic acid polymer of interest and a fourth labeled nucleic acid bearing a fourth fluorescent label, wherein said fourth fluorescent label has a fluorescence emission, said emission having a characteristic fluorescence lifetime.

85. The method according to claim 82, wherein said first sequencing reaction mixture and said second sequencing reaction mixture are members of a plurality of unique sequencing reaction mixtures, each reaction mixture comprising said nucleic acid polymer of interest and a unique labeled nucleic acid bearing a unique fluorescent

label, wherein said unique fluorescent label has a fluorescence emission, said emission having a characteristic fluorescence lifetime, said lifetime being different for each unique fluorescent label.

86. The method according to claim 77, wherein said sequencing reaction mixture further comprises one or more members selected from the group consisting of polymerases, exonucleases, endonucleases, deoxynucleotides, deoxynucleotide diphosphates, deoxynucleotide triphosphates, dideoxynucleotides, dideoxynucleotide diphosphates, dideoxynucleotide triphosphates, nucleotide analogs and nucleoside analogs and combinations thereof.

87. The method according to claim 77, wherein the labeled nucleic acids are members selected from the group consisting of nucleotides, nucleosides, nucleoside diphosphates, nucleoside triphosphates, dideoxynucleosides, deoxynucleotides, deoxynucleoside diphosphates, deoxynucleoside triphosphates, dideoxynucleosides, dideoxynucleotides, dideoxynucleoside diphosphates, dideoxynucleoside triphosphates, nucleotide analogs and nucleoside analogs and combinations thereof.

88. The method according to claim 77, wherein said nucleotide bearing a fluorescent label is a non-natural nucleotide.

89. The method according to claim 77, wherein said nucleotide bearing a fluorescent label comprises a base segment and a sugar segment and said fluorescent label is covalently attached to a segment which is a member selected from the group consisting of said base segment, said sugar segment and both said base segment and said sugar segment.

90. The method according to claim 77 further comprising, prior to step (b), producing a plurality of nucleic acid polymers complementary to a region of said nucleic acid polymer of interest.

91. The method according to claim 90, further comprising separating said complementary nucleic acid polymers into distinct populations, each of said populations consisting of nucleic acid polymers of about the same size.

92. The method according to claim 77, wherein said first fluorescent label is not a lanthanide chelate.

93. The method according to claim 77, wherein said nucleic acid polymer of interest is of a size of from about 2 bases to about 100,000 bases.

94. The method according to claim 93, wherein said nucleic acid polymer of interest is of a size of from about 100 bases to about 10,000 bases.

95. The method according to claim 94, wherein said nucleic acid polymer of interest is of a size of from about 300 bases to about 5000 bases.

96. The method according to claim 77 further comprising annealing said nucleic acid polymer of interest with a primer nucleic acid polymer.

97. The method according to claim 96, wherein said primer nucleic acid polymer comprises at least one labeled nucleic acid bearing a fluorescent label.

98. The method according to claim 97, wherein said fluorescent label is covalently attached to a labeled nucleic acid which is a member selected from the group consisting of the 3'-terminus, the 5'-terminus, an internal position and combinations thereof.

99. The method according to claim 78, wherein said first fluorescent label and said second fluorescent label have an emission maximum which occurs at substantially the same wavelength.

100. The method according to claim 78, wherein said first fluorescent label and said second fluorescent label have an emission maximum which occurs at a substantially different wavelength.

101. The method according to claim 77, wherein said fluorescent label has a fluorescence lifetime of from about 1 nanosecond to about 4000 nanoseconds.

102. The method according to claim 101, wherein said fluorescent label has a fluorescence lifetime of from about 1 nanosecond to about 1000 nanoseconds.

103. The method according to claim 102, wherein said fluorescent label has a fluorescence lifetime of from about 1 nanoseconds to about 100 nanoseconds.

104. The method according to claim 77, wherein said detecting is provided by a pulse method or a phase-modulation method.

105. The method according to claim 77, wherein said sequencing is performed using a microfluidic apparatus.

106. The method according to claim **c**, wherein said detecting is performed using a microfluidic apparatus.

107. The method according to claim 77, wherein said identifying by measuring is performed using a microfluidic apparatus.

108. The method according to claim 77, wherein said microfluidic apparatus comprises:

a glass or polymer body having therein, at least two intersecting channels fabricated into said surface of said substrate, at least one of said at least two intersecting channels having at least one cross-sectional dimension in the range of from about 0.1 μm to about 500 μm.

109. A method according to claim 77 further comprising performing a polymerase chain reaction.

110. An apparatus for distinguishing between a plurality of fluorescent species, wherein each of said fluorescent species has a fluorescence emission, said emission having a characteristic fluorescence lifetime comprising:

a microfluidic device comprising at least one microchannel;

a detector for detecting fluorescence emissions by the fluorescent species in the at least one channel; and,

a digital computer operably linked to the detector for determining fluorescence lifetimes of the fluorescent species.

111. The apparatus according to claim 110, wherein said microfluidic apparatus comprises a glass or polymer body having at least two intersecting channels

fabricated therein, at least one of said at least two intersecting channels having at least one cross-sectional dimension in the range of from about 0.1 μm to about 500 μm.

112. A sequencing reaction mixture comprising:

a first fluorescent label having a first fluorescent lifetime and a second fluorescent label having a second fluorescent lifetime, wherein said first fluorescent lifetime and said second fluorescent lifetime are different; and

a first nucleic acid and a second nucleic acid, said first nucleic acid and said second nucleic acid being members independently selected from the group consisting of nucleotides, nucleosides, nucleoside diphosphates, nucleoside triphosphates, dideoxynucleosides, deoxynucleotides, deoxynucleoside diphosphates, deoxynucleoside triphosphates, dideoxynucleosides, dideoxynucleotides, dideoxynucleoside diphosphates, dideoxynucleoside triphosphates, nucleotide analogs and nucleoside analogs.

113. A kit comprising a sequencing mixture according to claim 112.

114. A kit comprising a sequencing mixture according to claim 112 and a microfluidic device comprising a body having a plurality of microchannels fabricated therein.

115. A method of distinguishing between a plurality of fluorescent species, wherein each of said fluorescent species has a fluorescence emission, said emission having a characteristic fluorescence lifetime;

(a) electrokinetically separating each of said species;

(b) detecting each of said fluorescent species; and,

(c) identifying each of said fluorescent species by measuring said characteristic fluorescence lifetime.

116. Use of an apparatus or system of any of claims 1-52, 73 and 110 to practice a method selected from the methods of claims 53-72, 74-109 and 115.

117. Use of a sequencing reaction mixture of claim 112 or a kit of claim 113-114 for practicing a method selected from the methods of claims 53-72, 74-109 and 115.

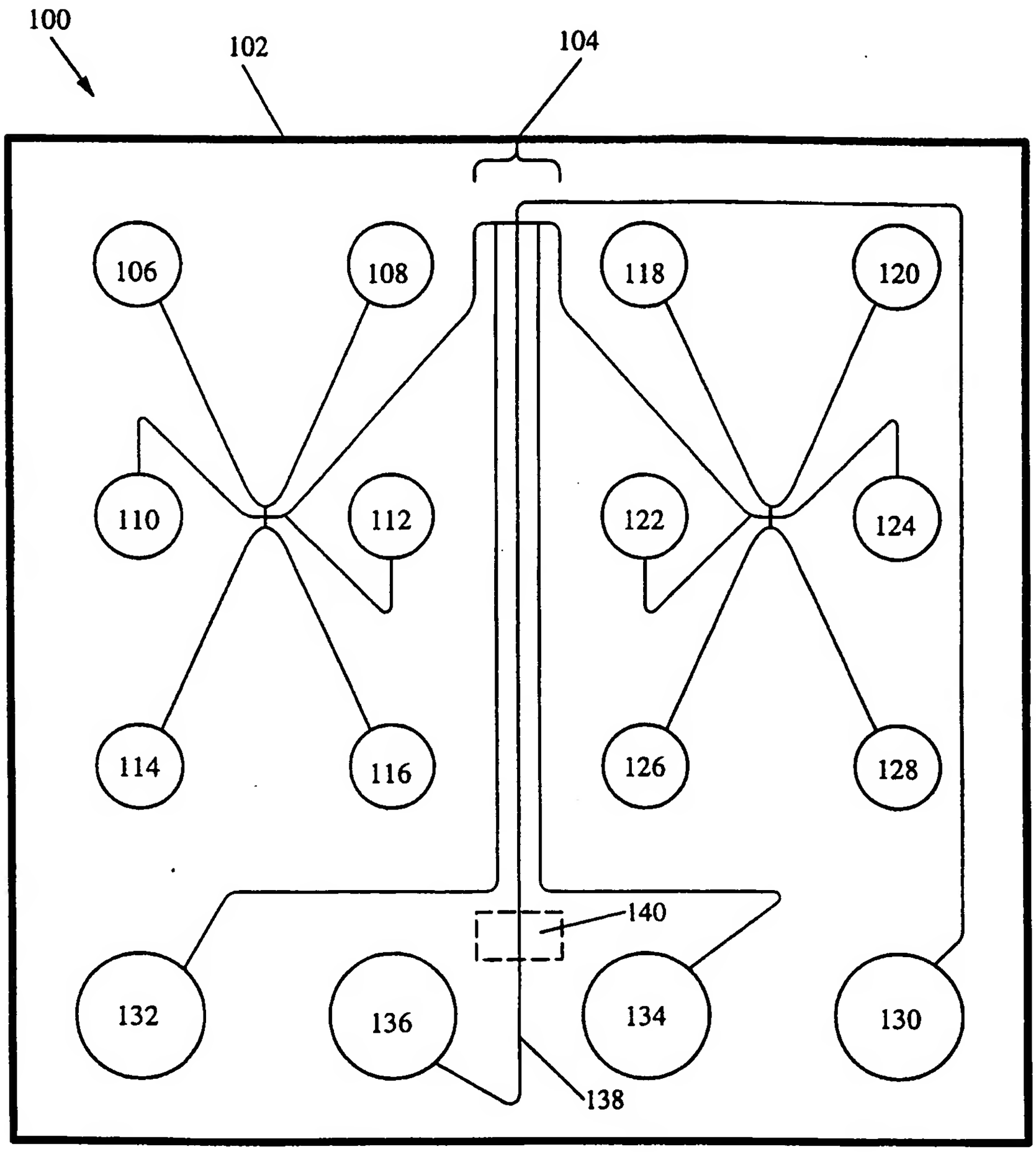

100
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Figure 1

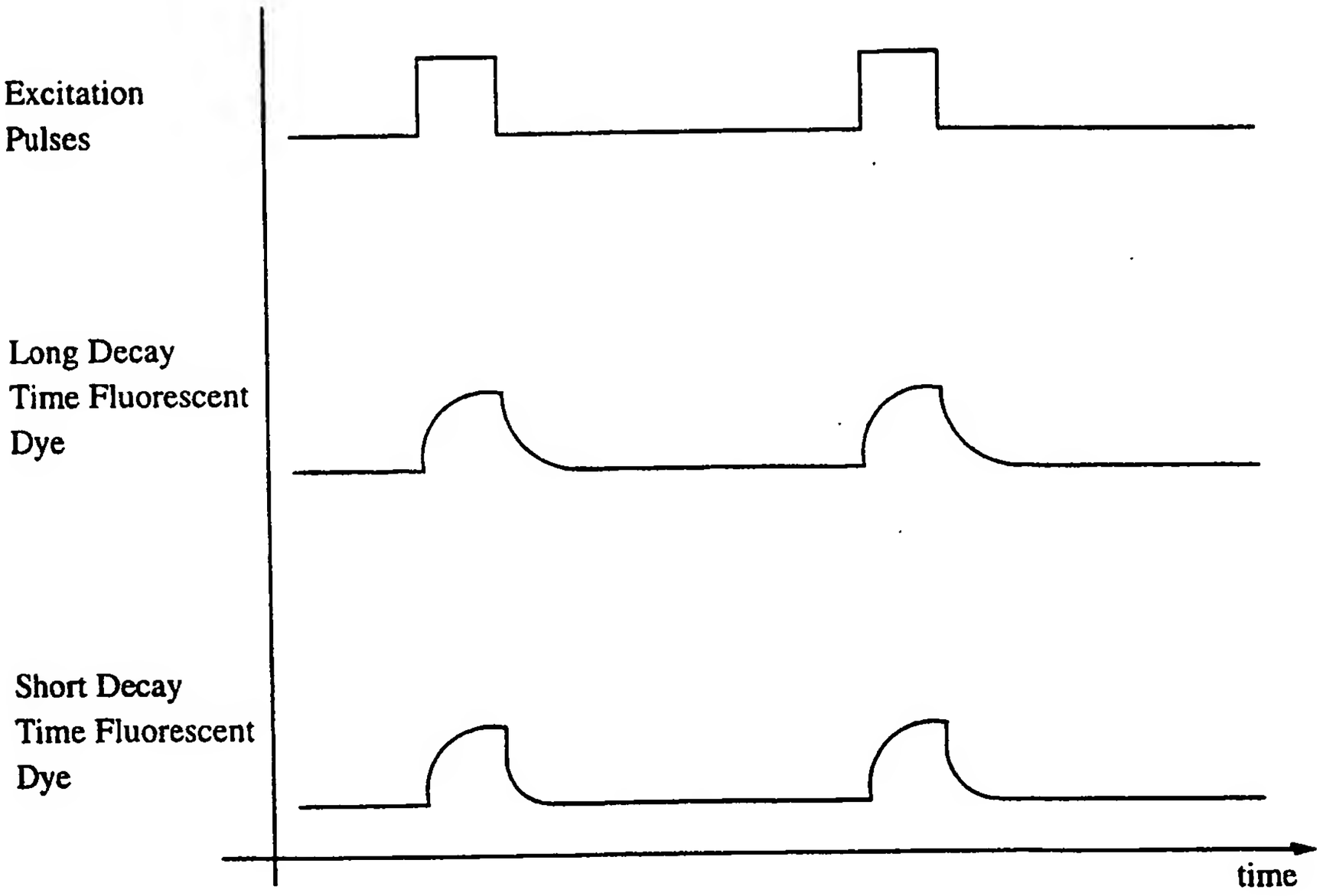
Excitation Pulses
Long Decay Time Fluorescent Dye
Short Decay Time Fluorescent Dye
time

Figure 2

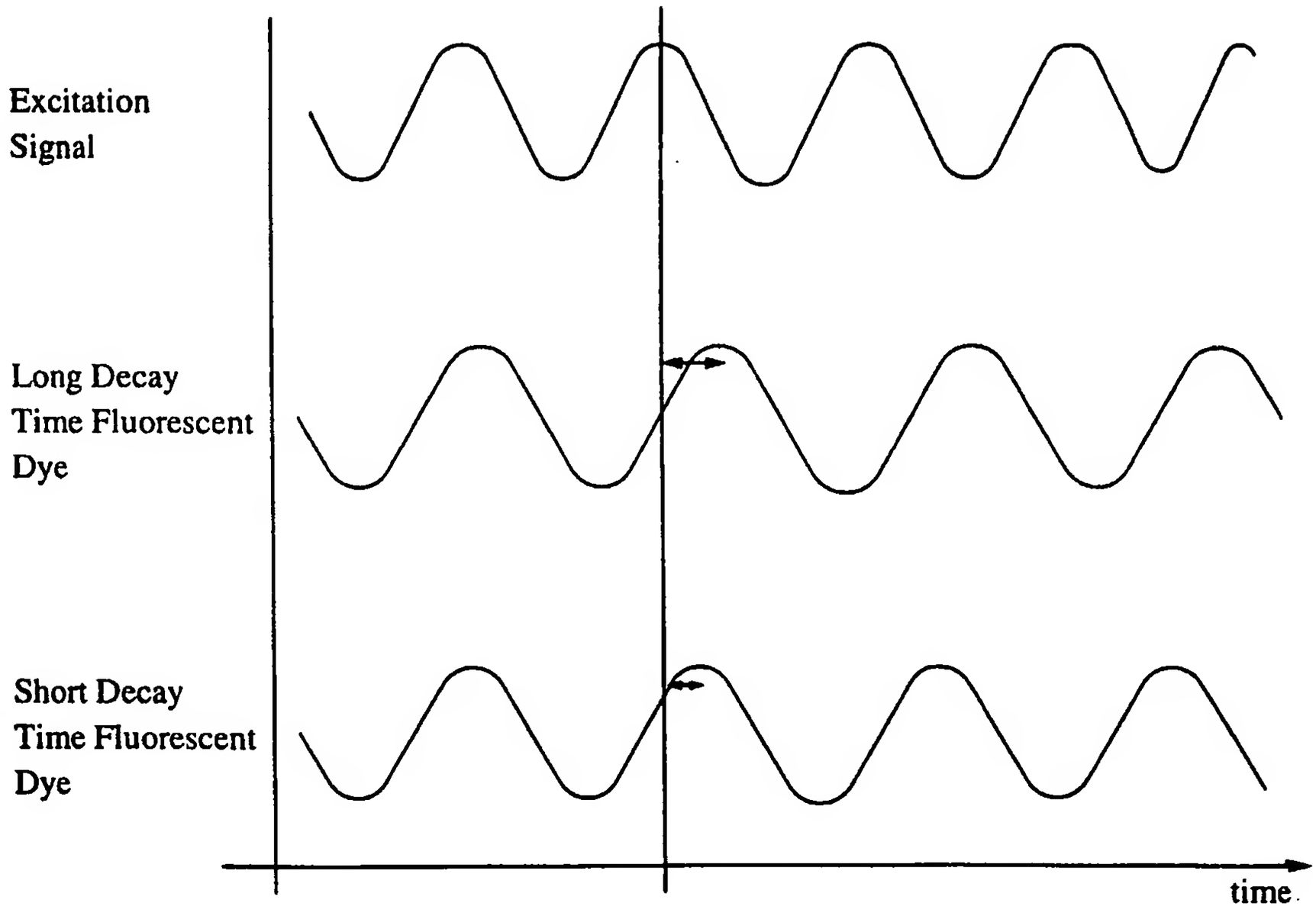
Excitation
Signal
Long Decay
Time Fluorescent
Dye
Short Decay
Time Fluorescent
Dye
time

Figure 3

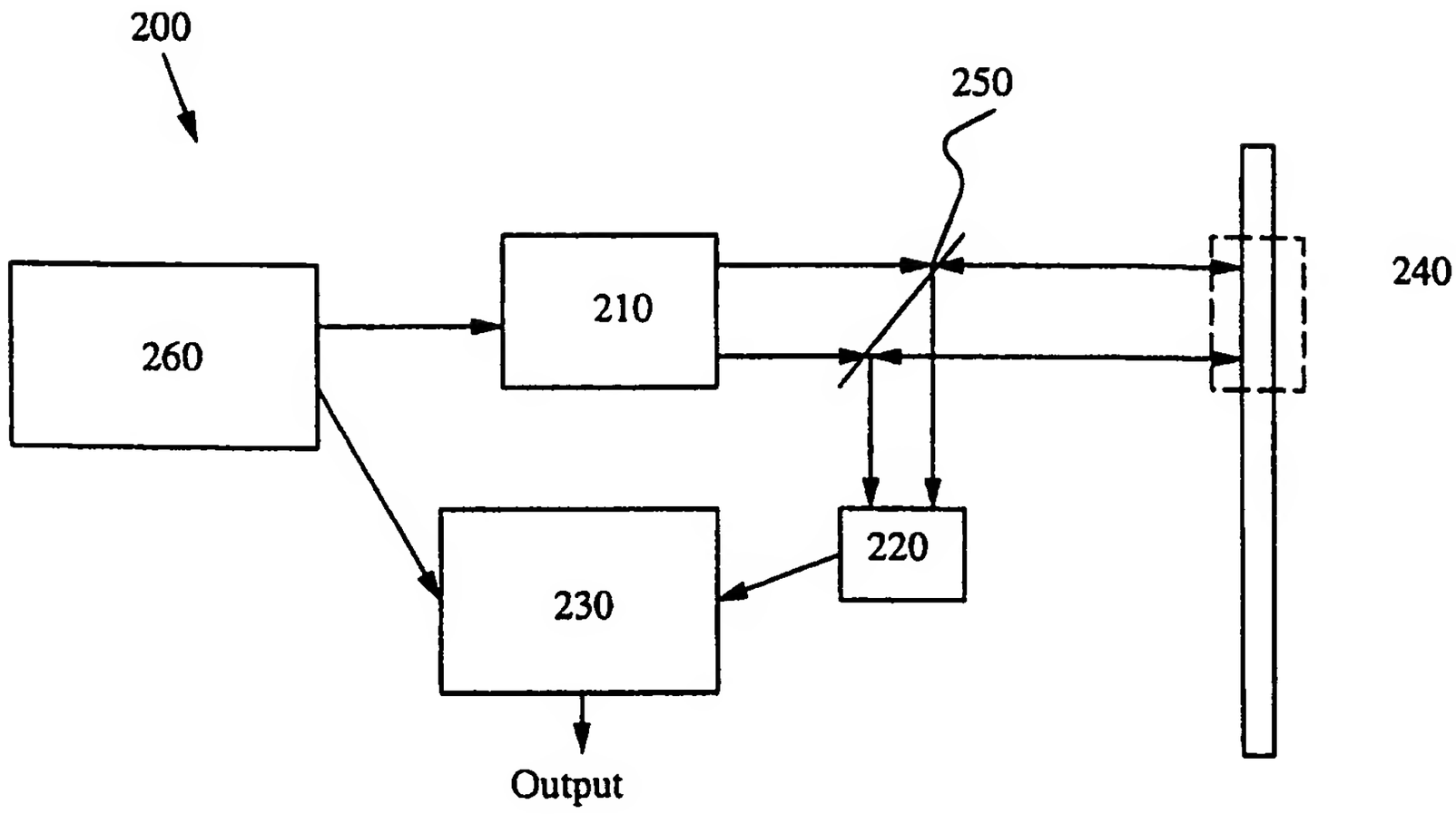

Figure 4

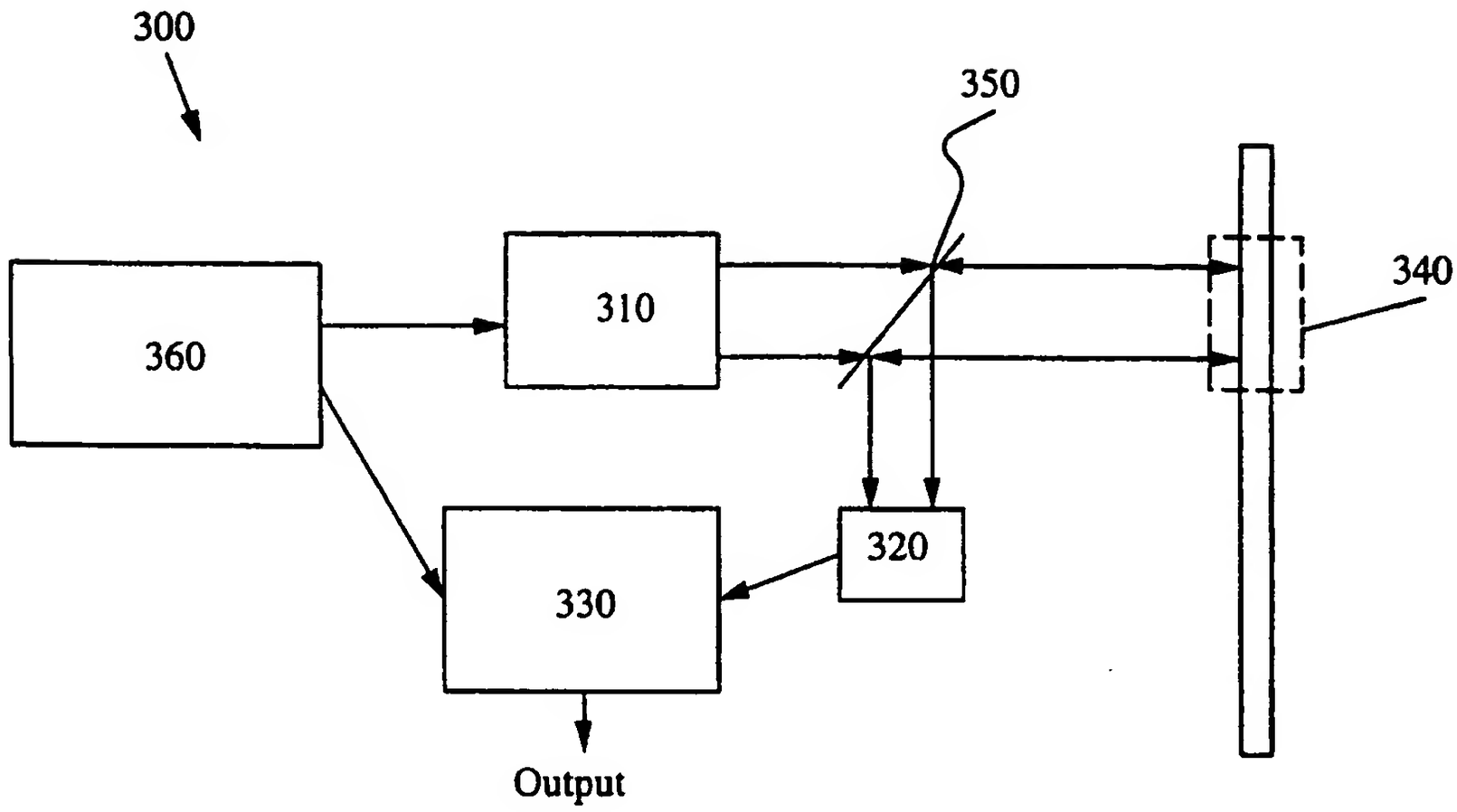

Figure 5

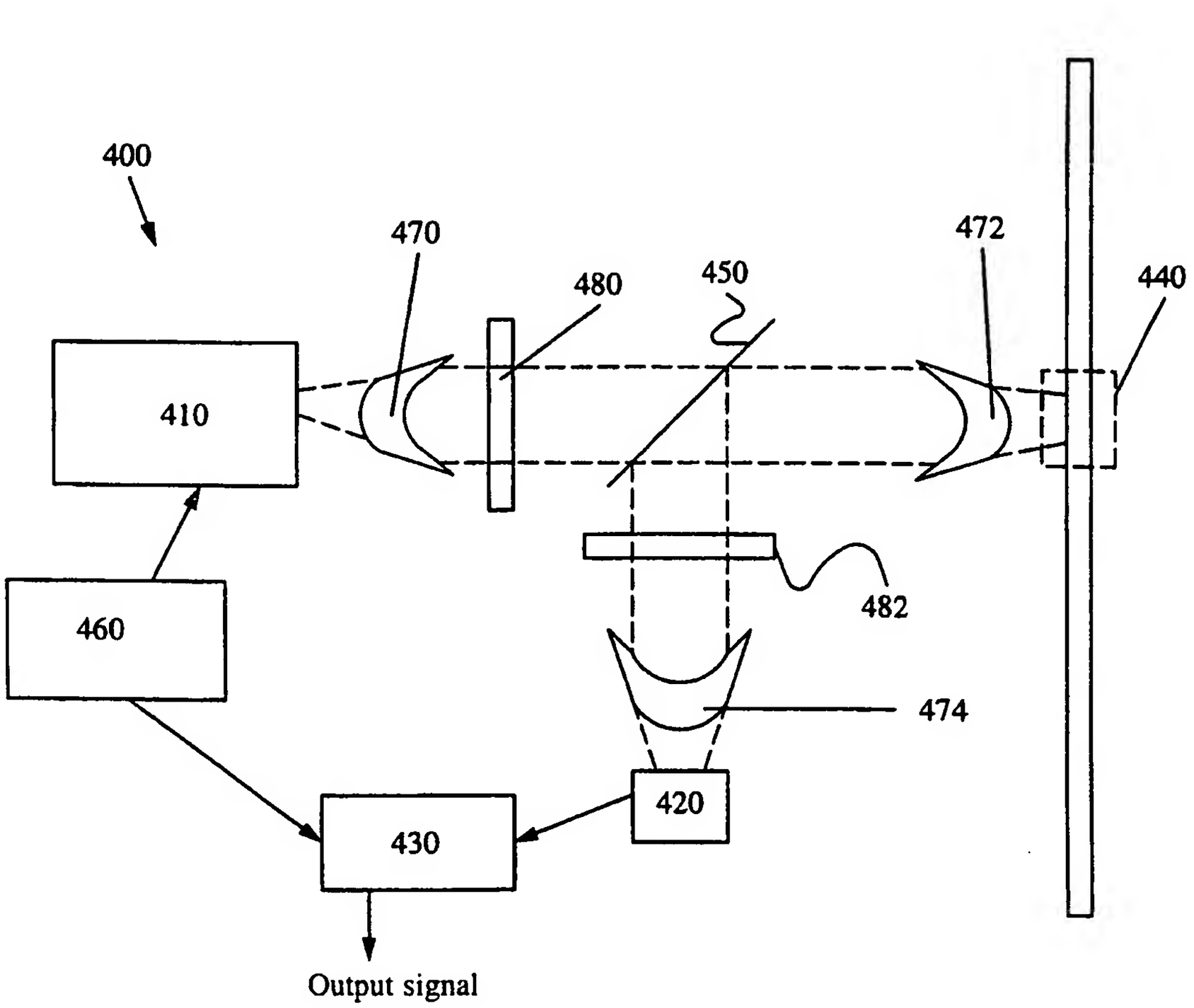

Figure 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/18294

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12Q 1/68; C07H 21/04
US CL :435/6, 91.2; 536/23.1, 24.33

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.2; 536/23.1, 24.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

USPAT, HCAPLUS, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,171,534 A (SMITH et al.) 12 December 1992, see entire document.	1-117
Y	US 4,849,513 A (SMITH et al.) 18 July 1989, see entire document.	1-117

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 20 DECEMBER 1999	Date of mailing of the international search report 11 JAN 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer SCOTT HOUTTEMAN Telephone No. (703) 308-0196

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